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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07K 14/78, 14/475, A61K 38/18, 38/39 — A1

(11) International Publication Number: **WO 00/55206**

(43) International Publication Date: 21 September 2000 (21.09.00)

(21) International Application Number: PCT/US00/07183

(22) International Filing Date: 17 March 2000 (17.03.00)

(30) Priority Data:
60/125,006 18 March 1999 (18.03.99) US

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(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ENDOTHELIAL CELL STIMULATION BY A COMPLEX OF FIBRONECTIN AND VASCULAR ENDOTHELIAL GROWTH FACTOR

(57) Abstract

The present invention pertains to isolated complexes containing the growth factor VEGF in association with the adhesion protein fibronectin or fragments thereof, and to methods of administering the complexes *in vitro* or *in vivo* to promote or induce endothelial cell migration, angiogenesis and wound healing.

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ENDOTHELIAL CELL STIMULATION BY A COMPLEX OF FIBRONECTIN AND VASCULAR ENDOTHELIAL GROWTH FACTOR

Field of the Invention

The present invention pertains to complexes containing vascular endothelial growth factor (VEGF) in association with the adhesion protein fibronectin or fragments thereof, and to methods of administering the complexes *in vitro* or *in vivo* to promote or induce endothelial cell migration, angiogenesis and wound healing.

Background of the Invention

The process of wound healing is complex and represents a serious medical problem affecting a large number individuals. Healing problems occur in dermal wounds such as decubitus ulcers, severe burns and diabetic ulcers and eye lesions including dry eye and corneal ulcer, as well as surgical wounds, and other wound related pathologies. One important aspect of wound healing is the migration of new cells from tissues surrounding a wound site which leads to the generation of new tissue having the proper population of cell types and tissue organization. In addition, central to the wound healing process is the growth of a functional blood supply system to cells in the healing zone. Without a regular flow of blood to the wound site there can be no substantial wound healing and restoration of health tissue. This is particularly true in the case of diabetic patients who have slow healing leg ulcers where the major reason for poor healing is insufficient blood supply. Promotion of angiogenesis and endothelial cell migration is also important for rapid patient recovery from vascular surgery involving the use of vascular prostheses (see U.S. Patent No. 5,880,090) and in the healing of ischemic areas of the heart.

Angiogenesis is the process by which new blood vessels are formed from a preexisting microvascular network, and takes place within the extracellular matrix.

This process is crucial for embryogenesis or wound healing, as well as for supporting the growth of solid tumors. Steps involved in the angiogenic process include the proteolytic remodeling of local vascular basement membrane and extracellular matrix, migration of endothelial cells into the matrix, endothelial cell proliferation, and the formation of tubular capillaries and a new basement membrane. In contrast, vasculogenesis involves the formation of new blood vessels by the *in situ* differentiation of mesodermal precursors to endothelial cells. Both are complex processes that involve coordinated regulation of endothelial cell proliferation, migration and differentiation (Risau and Flamme, *Ann. Rev. Cell Dev. Biol.* **11**:73-91 (1995); Folkman and Shing, *J. Biol. Chem.* **267**:10931-10934 (1992)).

Many kinds of molecules, particularly growth factors and extracellular matrix components, have been implicated in the complex processes of angiogenesis and vasculogenesis. Among these are vascular endothelial growth factor (VEGF), which specifically stimulates the proliferation of vascular endothelial cells, and fibronectin (FN), a high molecular weight glycoprotein of the extracellular matrix (Magnusson and Mosher, *Arterioscler Thromb. Vasc. Biol.* **18**:1363-70 (1998)).

VEGF has been shown to play a major role in vasculogenesis and angiogenesis by gene deletion studies (Ferrara et al., *Nature* **380**:439-442 (1996); Carmeliet, et al., *Nature* **380**:435-439 (1996)). Targeted disruption of the gene encoding VEGF receptor FLK-1 in mice resulted in failure of blood-island formation and endothelial differentiation (Shalaby et al., *Nature* **376**:62-66 (1995); Shalaby et al., *Cell* **89**:981-990 (1997)). Similarly, deletion of the FLK-1 kinase domain resulted in impaired vasculogenesis and angiogenesis (Hiratsuka, et al., *Proc. Natl. Acad. Sci. USA*, **95**:9349-9354 (1998); Fong et al., *Nature* **376**:66-70 (1995)). FLK-1 is also the first endothelial receptor tyrosine kinase to be expressed in the hemangioblast (Choi et al., *Development* **125**:725-732 (1998); Yamaguchi et al., *Development* **118**:489-498 (1993)). Recently it has been demonstrated that the hematopoietic progenitor cell $CD34^+$ can differentiate into endothelial cells and that VEGF was one of the critical factors for promoting the differentiation of $CD34^+$ cells into endothelial cells (Shi et al., *Blood* **92**:362-367 (1998); Asahara et al., *Science* **275**:964-967 (1997)). Together, these studies demonstrate that VEGF and its FLK-1 receptor are essential for the formation of new blood vessels.

Evidence is also accumulating that cell-extracellular matrix (ECM) interactions play an integral role in blood vessel development. The earliest ECM protein expressed in the embryo during vasculogenesis is fibronectin (FN) (Jiang et al., *J. Cell. Sci.* **107**:2499-2508 (1994); Risau et al., *Dev. Biol.* **125**:441-450

(1988)). Gene knockout studies have demonstrated that both FN and its major integrin receptor α5β1, are critical for vasculogenesis and angiogenesis in the developing embryo (George et al., *Blood* **90**:3073-3081 (1997); Yang et al., *Development* **119**:1093-1105 (1993)).

Collectively, these observations demonstrate that cell-ECM interactions are important for differentiation and tissue morphogenesis. ECM-integrin interactions not only mediate cell adhesion but can directly activate specific signalling pathways or integrate with growth factor induced signals to potentiate cellular processes (Miyamoto et al., *J. Cell. Biol.* **135**:1633-1642 (1996); Lee et al., *J. Biol. Chem.* **274**:22401-22408 (1999); Eliceiri et al., *J. Cell. Biol.* **140**:1255-1263 (1998)). Recent studies suggest that clustering of integrin and growth factor receptors is one potential mechanism through which signals integrate to amplify cellular processes (Jones et al., *J. Cell. Biol.* **139**:279-293 (1997); Schneller et al., *EMBO J.* **16**:5600-5607 (1997); Woodard et al. *J. Cell. Sci.* **111**:469-478 (1998)). How these receptors form specific associations to promote synergistic responses is still unclear.

Fibronectin exists as a soluble dimer, each subunit being a mosaic of repeating modules having a molecular weight of about 230 kDa. In its soluble state, it is nonreactive with adhesion receptors, but is highly reactive after the soluble form has polymerized onto specialized areas on cell surfaces or intracellular matrix. Fibronectin is believed to contribute to capillary formation both by supporting adhesion of endothelial cells that have migrated to the matrix, and by inducing the expression of other adhesion molecules needed for microtubule assembly (see, e.g., Nehls and Drenckhahn, *Microvasc. Res.* **50**:311-322 (1995)).

Many studies of angiogenesis have involved *in vitro* model systems that are amenable to manipulation. For example, Nehls and Drenckhahn (1995) describe an assay for quantifying endothelial cell migration and angiogenesis. This assay involves seeding endothelial cells on gelatin-coated microcarrier beads, then suspending the beads in a solution of fibrinogen. The fibrinogen is induced to polymerize, thereby entrapping the microcarriers in a three-dimensional fibrin matrix. These investigators observed that in response to either fibronectin, basic fibroblast growth factor or VEGF, cells migrated out of the matrix to form capillary-like structures.

Koolwijk et al. (*J. Cell Biol.* **132**:1177-1188 (1996)) devised a model system for studying the formation of tubular structures by human endothelial cells (i.e., angiogenesis). They cultured human foreskin microvascular endothelial cells (MVEC) on three-dimensional fibrin matrices in the presence of various growth

factors, and noted that bFGF and VEGF tested alone stimulated endothelial cell replication, but not tubule formation. However, when one or both of these growth factors were added simultaneously with TNF-alpha, tubule formation occurred, though cell replication was inhibited. They concluded that tubule formation required the presence of TNF-alpha, but that the process did not depend on cell replication.

Christenson and Stouffer (*Biol. Reprod.* 55:1397-1404 (1996)) reported that endothelial cells isolated from the corpus luteum could be cultured in the presence of VEGF on plates coated with various matrix proteins, including fibronectin and collagen I. They observed that, when serum was absent from the culture medium, VEGF stimulated endothelial cell DNA replication to a greater extent if culture plates were coated with fibronectin than with collagen I.

Spyridopoulos et al. (*J. Mol. Cell Cardiol.* **29**:1321-1330 (1997)) studied tumor necrosis factor-α-induced apoptosis in cultured endothelial cells in the presence of various growth factors. These authors observed that VEGF protected the cells against TNF-α-induced apoptosis, and that this protective effect was accompanied by the induction of fibronectin and β3 integrin expression. They concluded that the observed protective effect did not result from VEGF-induced cell proliferation, but suggested that the effect was related to the observed VEGF-induced augmentation of adhesion molecule expression.

Tezono et al. (*Thromb. Haemost.*, Suppl., Abstract PS-3020 (June, 1997)) observed that VEGF was released from fibrin clots during fibrinolysis, and proposed that, *in vivo*, VEGF is released from activated platelets, thereafter becoming entrapped in local blood clots. According to their hypothesis, VEGF is subsequently released from these blood clots to provide a mitogenic effect that aids in healing.

Vuori et al. (U.S. Patent No. 5,654,267) discloses that matrix compositions containing platelet derived growth factor (PDGF) and an integrin ligand such as vitronectin, promote cell migration and tissue regeneration in a synergistic fashion. However, the work of Vuori et al., did not establish whether the synergistic effects of the PDGF/vitronectin composition was due to the direct binding of the two molecules into a single complex prior to binding of vitronectin to an integrin cell surface receptor. In addition, Vuori et al., did not extend their studies to include a determination of whether other growth factors and extracellular matrix protein compositions, such as vascular endothelial growth factor and fibronectin might have effects on physiologically important cell migration events such as angiogenesis.

In view of the importance of angiogenesis and its central role in wound healing in the body and the formation and growth of tumors, it is medically desirable

that compositions and methods for both inducing and inhibiting angiogenesis are made available.

Summary of the Invention

Disclosed herein are previously unknown compositions and methods for promoting and inhibiting cell migration, cell differentiation and tissue regeneration that are associated with angiogenesis and vasculogenesis. Inventive compositions effective in stimulating cell migration are isolated complexes containing fibronectin (FN) and bound vascular endothelial growth factor binding domain (VEGF). In another embodiment of the invention the cell stimulatory composition comprises a peptide fragment of FN containing at least one VEGF binding site fused to a peptide encoding an arginine-glycine-aspartic acid (RGD) amino acid integrin binding domain, and bound VEGF. In this embodiment of the invention, the FN component of the composition usually contains an amino terminal 70 kDa peptide of FN. Alternatively, the FN component of the fusion peptide may have a carboxy terminal 40 kDa peptide of FN.

In yet another embodiment of the invention, the FN portion of the composition contains a fibronectin/vitronectin chimeric protein that is capable of stimulating cell migration. The FN portion of the chimeric protein is capable of forming tight associations with VEGF and the vitronectin (VN) portion is capable of binding platelet derived growth factor-BB (PDGF-BB). The chimeric FN/VN protein is preferably fused to a RGD integrin binding domain and bound with both VEGF and PDGF-BB. These compositions can be used directly to stimulate the migration of vascular endothelial cells, or they may be incorporated into a fibrin glue or other type of matrix, which in turn can be used to induce endothelial cell migration or cell differentiation. The matrix provides a physical support for cell migration and angiogenesis, as well as providing a means to deliver the fibronectin/VEGF or fibronectin/VEGF/vitronectin/PDGF compositions to sites in a human or animal in need of wound healing and angiogenesis. One preferred example of a matrix is fibrin glue, which when containing fibronectin/VEGF or fibronectin/VEGF/vitronectin/PDGF compositions may be applied to vascular grafts or other wound sites, such as burns, ulcers, and surgical wounds to faciliate healing. In addition, these compositions can be used to induce angiogenesis at ischemic areas of the heart.

Fibronectin compositions are also provided which lack a RGD integrin binding domain but retain the capacity to tightly bind to VEGF. In one embodiment of the invention, the FN component of the composition contains an amino terminal

70 kDa peptide of FN. In another embodiment, the FN component of the composition contains a carboxy terminal 40 kDa peptide of FN. Similarly, inventive chimeric proteins are provided which contain fibronectin and vitronectin fragments lacking a RGD integrin binding domain, yet are still capable of binding tightly to VEGF and PDGF. The fibronectin and fibronectin/vitronectin compositions that bind VEGF, yet do not bind to integrin are useful as inhibitors of the physiological responses induced by VEGF and PDGF, in particular, angiogenesis, cell migration and cell differentiation.

More specifically, a chimeric polypeptide is provided in which a VEGF-binding peptide of fibronectin is covalently joined to one end of a linker peptide that includes a RGD peptide sequence capable of binding to an integrin receptor. Exemplary VEGF-binding peptides of FN are an amino terminal 70 kDa peptide and a carboxy terminal 40 kDa peptide. In another embodiment of the invention, the chimeric polypeptide additionally comprises the platelet-derived growth factor (PDGF)-BB binding fragment of vitronectin, which is joined to the carboxy-terminus of the RGD integrin-binding domain by additional amino acids flanking the integrin-binding region. Thus, the second chimeric protein is capable of binding to an integrin cell surface receptor protein as well as to both VEGF and PDGF-BB. In yet another embodiment of the invention, the peptide sequences specifying the VEGF-binding fragment of fibronectin/integrin binding domain fusion and the VEGF-binding fragment of fibronectin/integrin binding domain/PDGF-BB binding fragment of vitronectin fusion are repeated multiple times. The above described chimeric proteins when bound to VEGF and PDGF-BB, may also be incorporated into fibrin glue or other matrix formulations to facilitate deliver of these compositions to sites in need of healing or stimulation of angiogenesis.

Further disclosed herein are methods of inhibiting the response of endothelial cells to VEGF and PDGF by exposing target cells to peptide fragments from the amino or carboxy termini of fibronectin that contain VEGF binding regions and fibronectin/vitronectin fusion proteins that contain both VEGF and PDGF binding regions but do not contain a RGD integrin binding domain. These methods can be applied, for example, to inhibit endothelial cells from migrating or to inhibit cells from differentiating into endothelial cells. In one embodiment of the inventive method the inhibitor FN peptide is a carboxy terminal 40 kDa peptide fragment. In another embodiment the method uses an amino terminal FN fragment of 70 kDa.

In yet another embodiment of the invention, a method is provided for inhibiting VEGF receptor function. First a VEGF/fibronectin fragment complex is

formed by contacting a carboxy terminal fragment of fibronectin with VEGF. The FN fragment of the complex lacks a RGD integrin binding domain. Usually the carboxy terminal fragment is about 40 kDa in size. The VEGF/fibronectin fragment complex is the contacted with a VEGF receptor to inhibit VEGF function. Usual VEGF receptors are VEGFR-1 and VEGFR-2. Inhibition of VEGF receptor function prevents binding of VEGF to the receptor and the signaling of cell migration. In addition, inhibition of VEGF receptor function prevents VEGF mediated induction of MAPK kinase activity.

Agents capable of blocking the formation or activity *in vivo* of fibronectin/VEGF complexes and vitronectin/PDGF complexes and or their association to cognate receptors can be used to inhibit the migration of cells as a means of inhibiting or preventing angiogenesis in tumors or to inhibit the formation of scar tissue or other undesired cell migration. Such blocking agent include peptides that mimic the protein domains in fibronectin or VEGF and vitronectin or PDGF that mediate their respective associations, including synthetically modified peptides.

Brief Description of the Drawings

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same become better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIGURE 1 illustrates microvessel endothelial cell (MVEC) adhesion to each of several matrix proteins when cultured in the presence of 10 ng/ml VEGF.

FIGURE 2 compares the effects on MVEC migration of several different adhesion proteins when tested alone or together with VEGF.

FIGURE 3 graphically illustrates measurements of MVEC migration in the presence of various concentrations of VEGF with or without the simultaneous addition of fibronectin to the medium.

FIGURE 4A shows a diagrammatic representation of the fibronectin molecule (not to scale), with the text above the diagram denoting various binding regions, including regions that bind to the indicated matrix proteins.

FIGURE 4B illustrates the capacity of various fibronectin fragments to bind with VEGF in a filter-binding assay.

FIGURE 5 graphically presents SPR analysis of VEGF binding to the N-terminal 70 kDa FN fragment. Representative trace shows that maximal VEGF binding to the 70 kDa N-terminal FN fragment was achieved within 1 minute.

FIGURE 6A graphically illustrates the effect of VEGF/ECM protein complexes on endothelial cell migration. Data are presented as mean ± standard error of the mean.

FIGURE 6B graphically illustrates the effect of ECM protein and VEGF on hematopopoietic $CD34^+$ cell differentiation into endothelial cells.

FIGURE 7 graphically illustrates the effects of VEGF/FN (closed circles), VEGF/120 kDa FN fragment (triangles) and VEGF/vitronection (open circles) on MAPK activity.

FIGURE 8A graphically illustrates inhibition of VEGF-induced endothelial cell migration in the presence or absence of the amino terminal FN 70 KDa and carboxy terminal FN 40 kDa fragments. Data are represented as the mean ± standard error of the mean.

FIGURE 8B graphically illustrates FN fragment inhibition of ^{125}I-VEGF binding to VEGFR-1. Data are represented as the mean ± standard error of the mean.

FIGURE 9 graphically illustrates measurements of MVEC migration in the presence of various fibrin glue compositions with or without fibronectin and VEGF. In FIGURE 9, A represents fibrin glue minus fibronectin, B represents fibrin glue minus fibronectin plus VEGF (50 ng/ml), and C represents fibrin glue plus fibronectin and VEGF (50 ng/ml); and

Detailed Description of the Preferred Embodiment

Provided herein are compositions containing a complex formed between VEGF and an adhesion protein, such as fibronectin, laminin or fibrin. It is shown here that an affinity exists between VEGF and fibronectin, such that when the two proteins are mixed together in solution, they form a tight complex that retains the physiological activity of VEGF. This VEGF/FN complex is capable of stimulating the migration of vascular endothelial cells to a greater extent than when the cells are exposed to VEGF alone. In addition, it has been shown that the effects of VEGF on endothelial cell migration can be enhanced by mixing the VEGF with fibrin, laminin or a mixture of fibronectin, fibrin and laminin. Further, the inventive composition of VEGF and FN induces differentiation of $CD34^+$ hematopoietic cells into endothelial cells as compared to VEGF alone or VEGF complexes with other extracellular matrix (ECM) proteins.

In one aspect the present invention provides an isolated complex of VEGF and fibrinogen. As used herein the term "isolated complex formed between VEGF and FN" refers to any isolated complex whereby FN and VEGF are bound to each other and the complex is greater than about 50% pure. Usually the VEGF/FN

complex is greater than 80% pure, and more often greater than 95% pure. The inventive isolated VEGF/VN complex can also be incorporated into a fibrin glue. A fibrin glue is a biological adhesive consisting of highly concentrated human fibrinogen, thrombin and factor VIII (see, for example, Suzuki et al., *Arch. Surg.* **130**:952-55 (1995)). In practice, the fibrinogen is mixed with the other two components during the application of the glue, so that fibrin forms *in situ* upon exposure to the thrombin and factor VIII. Additional biologically active molecules, such as the VEGF complexes of the present invention, can be incorporated into such a glue. This mode of use will ensure the slow and continuous release of the VEGF complexes at the site of glue application. The fibrin glue also provides an easy to use delivery vehicle for directing the inventive compositions to sites in need of wound healing or angiogenesis. Yet a further advantage of the fibrin glue compositions is that the glue matrices provide a temporary support structure onto which migrating cells can attach themselves.

The VEGF/adhesion protein complexes of the present invention, such as a fibronectin/VEGF complex, may also be incorporated into other types of matrices other than fibrin glue, including, but not limited to matrices made from hyaluronic acid, chondroitin sulphate, agarose and collagen. As used herein, the term "matrix" refers to any biocompatible solid or non-solid support which functions as a scaffold for tissue repair or angiogenesis and is complexed or conjugated with fibronectin and VEGF. A wide variety of such matrices and methods for complexing protein components to them are fully disclosed in U.S. Patent No. 5,654,267. Suitable matrix materials for use in the practice of the invention include, for example, hyaluronic acid, chondroitin sulfate, heparin, heparin sulfate, polylactate, polyglycolic acid, starch and collagen. Effective concentrations of fibronectin and VEGF for incorporation into a matrix will vary depending upon the composition of the matrix and the particular purpose. Such concentrations may be determined using assays and techniques disclosed herein as well as other assays and methods that are well known in the art. For example, a variety of matrices can be assembled, each containing a different ratio of fibronectin to VEGF. Each combination is then evaluated for its ability to support cell attachment and migration using *in vitro* and *in vivo* assays. Generally, the biologically active molecules of the present invention will be present in the above matrix compositions in a concentration range of about 50 ng/ml to about 1 mg/ml.

In addition, disclosed herein are chimeric polypeptides containing a peptide fragment of fibronectin which is capable of binding to VEGF that is covalently

joined at its carboxyl terminus to a RGD peptide capable of binding to an integrin receptor. An additional chimeric polypeptide is provided that contains the fibronectin/integrin receptor binding site fusion but further includes a peptide fragment of vitronectin which is capable of binding to platelet-derived growth factor-BB (PDGF-BB) that is covalently joined at its amino terminus to said fibronectin/integrin receptor binding site fusion protein. PDGF-BB is a particular isoform of platelet-derived growth factor that has been shown to have a synergistic effect upon cell migration and wound healing when combined with vitronectin, an integrin ligand (See U.S. Patent No. 5,654,267).

The inventive chimeric polypeptides, in some embodiments of the invention, also include an arginine-glycine-aspartic acid (RGD) amino acid sequence capable of binding to an integrin receptor. The RGD sequence has been shown to be essential for binding to $\alpha_v\beta_3$ integrin and other integrin molecules. Pierschbacher et al. ((1984) *Proc. Natl. Acad. Sci.* **81**:5985-5988) showed that the amino acid sequence RGD is critical for the role of cell attachment activity essential for the mechanism of many integrins. The binding of $\alpha_v\beta_3$ and other integrins to RGD sequences is reviewed in Ruoslahti et al. ((1986) *Cell* **44**:517-518) and discussed in Smith et al. ((1990) *J. Biol. Chem.* **265**:12267-12271); Ruoslahti et al. ((1987) *Science* **238**:491-497); Humphries, (1990) *J. Cell Sci.* **97**:585-92); Ruoslahti, (1991) *J. Clin. Invest.* **87**:1-5). Residues adjacent to the RGD can also play a role in integrin binding as shown by Pierschbacher et al. ((1987) *J. Bio. Chem.* **262**:17924-17928). However, the Arg-Gly-Asp sequence is not the only integrin recognition motif used by adhesive ligands. Another integrin $\alpha_4\beta_1$ binds the variable region (CS1) of fibronectin via the sequence Leu-Asp-Val (LDV). The tripeptide Leu-Asp-Val is the minimal sequence within CS-1 capable of supporting hematopoietic cell adhesion or of inhibiting integrin $\alpha_4\beta_1$-mediated cell binding to fibronectin (Komoriya et al., (1991) *J. Biol. Chem.* **23**:15075-15079; Wayner et al., (1992) *J. Cell Biol.* **116**:489-497).

A chimeric peptide of the present invention that is useful in stimulating cell migration and cell differentiation includes a RGD integrin binding peptide and is attached covalently at one end to a VEGF-binding fragment derived from fibronectin. Preferably the chimeric peptides are composed of a 70 kDa fragment from the amino terminus of FN or a 40 kDa fragment from the carboxy terminus of FN. In other embodiment the chimeric protein additionally comprises a PDGF-BB binding region of vitronectin that is covalently joined to the VEGF-binding fragment of fibronectin and the RGD integrin binding peptide sequence. Chimeric molecules may include

several iterations and combinations of these three motifs. In addition, such chimeric peptides may contain multiple copies of each of the three binding domains. Furthermore, the amino acids surrounding the RGD peptide may be selected such that the inventive chimeric proteins will bind to $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_5\beta_1$ or any other integrin that will promote angiogenesis. The inventive fibronectin/vitronectin chimeric proteins bound to VEGF and PDGF can also be incorporated into fibrin flue and other types of matrices in order to facilitate delivery of the compositions to tissue sites in need of stimulation of cell migration and angiogenesis.

In yet another embodiment of the invention, a method is provided for promoting angiogenesis whereby cells are contacted with a composition comprising an isolated complex formed between VEGF and fibronectin. The above described chimeric proteins having a RGD integrin binding site can also be used to practice the inventive method. Usually the angiogenesis promoting composition further contains a matrix such as fibrin glue.

The subject invention further provides a method of inhibiting the physiological responses of vascular endothelial cells to vascular endothelial growth factor and/or platelet derived growth factor. In this aspect of the invention, cells are contacted with a portion or fragment of the fibronectin molecule that binds VEGF but is lacking the RGD amino acid domain necessary for binding to an integrin receptor. The presence of a LDV integrin binding site does not affect the inhibitory activity of the VEGF/FN fragement complex. Alternatively, endothelial cells are exposed to a portion or fragment of the vitronectin molecule that contains a PDGF-BB-binding domain capable of binding PDGF-FF but is lacking the RGD integrin binding domain. In yet another embodiment of this invention the two growth factor-binding domains, both lacking their respective RGD integrin receptor binding regions, are fused together to form a chimeric protein capable of binding to both VEGF and PDGF-BB.

In another aspect of the invention, a method of inhibiting VEGF receptor function is provided. First a VEGF/fibronectin fragment complex is formed by contacting a carboxy terminal fragment of fibronectin with VEGF. Usually the carboxy terminal fragment is about 40 kDa in size. The VEGF/fibronectin fragment complex is the contacted with a VEGF receptor to inhibit VEGF function. Usual VEGF receptors are VEGFR-1 and VEGFR-2. Inhibition of VEGF receptor function prevents binding of VEGF to the receptor and the signaling of cell migration. In addition, inhibition of VEGF receptor function prevents VEGF mediated induction of MAPK kinase activity.

The biological response of VEGF is mediated through its high affinity VEGF receptors which are selectively expressed on endothelial cells during embryogenesis (Millauer et al. *Cell* 72:835-846 (1993)) and during tumor formation. VEGF receptors typically are class III receptor-type tyrosine kinases characterized by having several, typically 5 or 7, immunoglobulin-like loops in their amino-terminal extracellular receptor ligand-binding domains (Kaipainen et al., *J. Exp. Med.* **178**:2077-2088 (1993)). The other two regions include a transmembrane region and a carboxy-terminal intracellular catalytic domain interrupted by art insertion of hydrophilic interkinase sequences of variable lengths, called the kinase insert domain (Terman et al., *Oncogene* 6: 1677-1683 (1991)). VEGF receptors include FLT-1 homolog from human (Flt-1 homolog gene sequenced by Shibuya et al. (*Oncogene* 5:519-524 (1990)); KDR, described in PCT/US92/01300, filed Feb. 20, 1992, and in Terman et al. (*Oncogene* 6:1677-1683 (1991)); and FLK-1 (murine Flk-1 gene sequenced by Matthews et al. (*Proc. Natl. Acad. Sci.* **88**:9026-9030 (1991)). The KDR receptor from humans is also known as VEGFR-1 (Neufeld et al. *FASEB J.* 13:9-22 (1999)).

As used herein the term "VEGF receptor" refers to any mammalian protein having substantially the same amino acid sequence as VEGFR-1,VEGFR-2, FLK-1 and FLT-1. "Substantially the same" amino acid sequence is defined herein as a sequence with at least 70%, preferably at least about 80%, and more preferably at least about 90% homology to another amino acid sequence, as determined by the FASTA search method in accordance with Pearson and Lipman ((1988) *Proc. Natl. Acad. Sci.* **85**:2444-2448).

VEGFR-1 is a human form of a VEGF receptor having MW 180 kDa. FLK-1 is the murine homolog of VEGFR-1. FLT-1 an additional murine form of VEGF receptor different from, but related to, the VEGFR-1/FLK-1 receptor. The human homolog to the murine FLT-1 receptor is VEGFR-2. Other VEGF receptors that can be inhibited by certain compositions of the present invention include those that can be cross-link labeled with VEGF, or that can be co-immunoprecipitated with VEGFR-1 (MW 180 kDa). Some known forms of these VEGF receptors have molecular weights of approximately 170 kDa, 150 kDa, 130-135 kDa, 120-125 kDa and 85 kDa. See, for example, Quinn et al. (*Proc. Nat. Acad. Sci* 90:7533-7537 (1993); Scher et al. (*J. Biol. Chem.* 271:5761-5767 (1996)). The VEGF receptor is usually bound to a cell, such as an endothelial cell. The VEGF receptor may also be bound to a non-endothelial cell, such as a tumor cell. Alternatively, the VEGF receptor may be free from the cell, preferably in soluble form.

All of the physiologically inhibitory fibronectin fragments, vitronectin fragments and FN/VN chimeric fragment proteins are capable of binding to VEGF, PDGF-BB or both VEGF and PDGF-BB molecules respectively, thereby preventing the VEGF and/or PDGF-BB molecules from binding to their biologically active receptors on the cell surface and initiating their cognate biological responses. In additional, all of the inhibitory VN and VN/PDGF-BB proteins lack a RGD integrin binding site. In one embodiment of the invention a carboxy terminal VEGF binding domain of FN is provided which contains a VEGF binding domain capable of binding to VEGF but does not contain a RGD integrin binding domain. In a preferred embodiment, the carboxy terminal fibronectin fragment used as an inhibitor of VEGF responses has a molecular weight of about 40 kDa. In particular, the fibronectin and vitronectin fragments can be used to inhibit endothelial cell migration and proliferation.

The following examples are offered to illustrate the present invention and are not intended to limit the means and ways by which the improvements can be applied.

Example 1

Adhesion of MVECs to Various Matrix Proteins

For these experiments, 48-well plates were precoated with various matrix proteins by adding 250 µl of phosphate buffered saline (PBS) containing 2 µg/ml of each test protein, then incubating overnight at 4°C. Prior to the experiments, the PBS and test protein solution was removed from the plates, and the plates were washed with 500 µl of MCDB 131 culture medium obtained from Clonetics (San Diego, CA). Primary cultures of microvessel endothelial cells (MVECs) were obtained from Clonetics (San Diego, CA) and 2 X 10^6 cells were cultured at 37°C in MCDB 131 medium containing 5% fetal bovine serum (FBS) until confluent. Monolayers of cultured MVECs were trypsinized, then washed twice in 10 ml of MCDB 131 containing 0.25% bovine serum albumin (BSA) (assay medium), and resuspended at 1x10^6 cells/ml in assay medium. Cells were added (2 x 10^4/well), and incubated for two hours at 37°C, and the extent of cell adhesion was evaluated by staining with crystal violet and absorbence read at 660 nm as described in Example 2. The data presented in FIGURE 1 show that MVECs bind equally well to each of the matrix proteins tested.

Example 2

Stimulation of MVEC Migration by VEGF and Matrix Proteins

Experiments were conducted to determine the capacity of various proteins, including several extracellular matrix proteins, to induce the migration of MVECs in

the presence of 10 ng/ml of VEGF. Cells were isolated and cultured as described in Example 1. To address this question, the chemotactic activity of MVECs was measured in Transwell migration assays in the presence of VEGF and one of the following proteins: albumin; fibronectin; vitronectin; fibrin; laminin; collagen I; or collagen IV.

Cell migration assays were performed with minor modifications essentially as described by Leavesley et al., *J. Cell. Biol.*, **121**:163-170 (1993), which is hereby incorporated by reference. In brief, cells were placed into Transwells (Costar Corp., Cambridge, MA), which are inserts that fit into the wells of 24-well plates and whose bottom surface consists of a porous membrane through which cells can migrate. When the Transwell is inserted into a well, cells in the upper compartment can be induced to migrate through the membrane by chemo-attractants present in the lower chamber. Transwells used in these assays had 8 μm pore membranes.

Primary cultures of MVECs were isolated as described in Example 1. Monolayers of cultured MVECs were trypsinized, then washed twice in MCDB 131 containing 0.25% BSA (assay medium), and resuspended at $1x10^6$ cells/ml in assay medium.

Each of the proteins to be tested (0.25 ml of 2 μg/ml in assay medium) was added to the wells of 24-well tissue culture plates (microwells), and incubated for 20 minutes at 37°C. Control wells received 0.25 ml of the same assay solution without the test protein. A solution containing VEGF (0.5 ml of a 10 ng/ml solution) was then added to the wells, and incubated for a further 10 minutes to permit the spontaneous formation of protein/VEGF complexes. One hundred μl of the MVEC suspension (at $1x10^6$ cells/ml) were added to each Transwell, and the Transwells were inserted into the microwells. Cell migration was measured after 6 hours of incubation at 37°C. To measure the number of cells that had migrated from the upper to the lower chamber, the non-migrant cells were removed from the upper face of the Transwell membrane with a cotton swab, and the migrant cells, i.e., those attached to the lower face of the membrane, were fixed and stained with 0.1% crystal violet. Dye was eluted with 10% acetic acid, and the numbers of cells were quantified by reading the absorbence at 600 nm.

FIGURE 2 shows that when fibrin, laminin or fibronectin was present together with VEGF in the lower chamber, cell migration was stimulated to a degree significantly higher than was observed when these proteins or VEGF were added alone. When fibronectin was used together with VEGF, the amount of migration observed exceeded the amount that would have been expected if the effects of the

different molecules were merely additive (see FIGURE 2). This observation suggests that VEGF acts synergistically with fibronectin in stimulating cell migration.

The ability of fibronectin to stimulate cell migration was investigated further by determining whether the combined effects of fibronectin and VEGF on MVEC migration was dependent on VEGF concentration. The results of a dose-response test, graphically illustrated in FIGURE 3, showed that in the presence of a fixed concentration of fibronectin (2 μg/ml), the amount of cell migration increased with increasing VEGF concentration up to a VEGF concentration of about 50-60 ng/ml. This result suggests that the enhanced cell migration observed in the presence of fibronectin and VEGF may result from the formation of a specific complex.

Example 3

Binding of VEGF to Extracellular Matrix Proteins

To assess their ability to form a complex with VEGF, several extracellular matrix proteins were tested for binding to VEGF on nitrocellulose membrane. The proteins tested were fibronectin, vitronectin, laminin, fibrinogen, fibrin, collagen I, collagen IV and collagen IV. To prepare the nitrocellulose membrane, 5 micrograms of each protein were loaded under vacuum onto nitrocellulose membranes using a slot-blot apparatus. The membranes were then incubated for one hour at room temperature with a solution of 20 ng/ml VEGF in PBS. After the binding step, the membranes were washed with PBS to remove unbound VEGF, and bound VEGF was quantified by incubating the filters with antibody against VEGF.

Using the above-described binding assay, fiberin, fibronectin, laminin, and fibrinogen were all found to bind detectable amounts of VEGF (data not shown). However, the amount of VEGF bound to fibronectin was greater than the amount of VEGF bound to any of the other proteins that bound VEGF, suggesting that fibronectin binds very tightly with VEGF.

In addition, two of the isoforms, i.e., splicing variants of VEGF, were tested in this same assay. Four such isoforms are known; these have 206, 185, 165 and 121 amino acids. All except the $VEGF_{121}$ variant are known to bind to heparin. The two isoforms selected for testing were $VEGF_{165}$ and $VEGF_{121}$. Both bound equally well in the binding assay, thus indicating that heparin binding is not an aspect of the cell migration observed in Examples 2 and 3.

Example 4

Binding of PDGF-BB to Extracellular Matrix Proteins

To determine the specificity of PDGF binding to vitronectin, plasma proteins were tested for their ability to bind PDGF-BB in the same fashion as described in Example 3 for VEGF. Proteins tested were vitronectin (as a positive control) plasminogen, fibronectin, fibrinogen, and serum albumin. Five micrograms of each protein were loaded under vacuum onto nitrocellulose membranes using a slot-blot apparatus. The membranes were then incubated for one hour at room temperature with radiolabeled PDGF-BB (NEN Life Science, Boston, MA) in PBS. The membranes were then washed with PBS to remove unbound PDGF-BB, and bound PDGF-BB was quantified using a gamma counter. Results indicated that only vitronectin mediated the binding of the labeled PDGF-BB to nitrocellulose filters.

Example 5

Analysis of VEGF Binding to Fragments of the Fibronectin Polypeptide

Fragments of the fibronectin polypeptide were tested individually in binding assays in order to identify the VEGF binding domain within the fibronectin molecule. FIGURE 4A presents a diagrammatic representation of the fibronectin protein molecule (not to scale), with the various protein domains denoted, including regions that bind to the cellular matrix proteins. The fibronectin fragments that were tested for their capacity to bind to VEGF are illustrated in FIGURE 4B, and were obtained from Sigma (St. Louis, MO) or Life Technologies (Grand Island, NY). Each fragment was bound to a nitrocellulose membrane as described above in Example 3. The relative binding observed with each fragment is shown in FIGURE 4B, using a minus sign or one or more plus signs to indicate the degree of binding that was observed. The strongest VEGF binding was observed using a carboxy terminal 40 kDa fragment. An amino terminal 70 kDa fragment also exhibited strong binding to VEGF. Two smaller fragments derived from the amino terminal 70 kDa fragment also bound VEGF, though not as strongly. The 120 kDa fragment from the middle portion of fibronectin exhibited the least amount of binding. From these results, it appears that their are two VEGF-binding domains located in fibronectin. One VEGF binding domain is located near the amino terminus of the molecule, and is entirely contained within the 70 kDa fragment shown in FIGURE 4B. The other VEGF binding domain is located on the carboxy terminal portion of fibronectin.

30 kDa and a 40 kDa fragments derived from the amino terminal 70 kDa fragment of fibronectin were tested also. These fragments bound VEGF, but to a lesser degree than the intact 70 kDa fragment, thus suggesting that the 30 kDa and 40 kDa fragments encompasses part but not all of the amino terminal VEGF binding region.

To confirm the observations of the above slot blot assays, the interaction of VEGF with the amino terminal FN 70 kDa peptide was performed in real time by surface plasmon resonance analysis (SPR) using BIACORE® methodology (Biacore Inc., Piscataway NJ). FN fragments were coupled to CM5 dextran chips by amine coupling chemistry according to Biacore Inc. instructions. The reference cell (serving as a control) had immobilized mouse IgG, and all binding curves were subtracted for non-specific binding to that reference. VEGF was used at two different concentrations (1.3 μM and 2.6 μM). Using the SPR method VEGF was found to bind to the 70 kDa peptide (FIGURE 5). Binding was maximal within 1 minute. Similarly, VEGF also bound to the carboxy terminal 40 kDa peptide. As expected, VEGF binding was not observed with the 120 kDa peptide (data not shown). Collectively, these studies demonstrate that FN possesses two binding sites for VEGF, one located at its N-terminus and the other at its C-terminus.

Example 6

VEGF/FN Complex Formation

Given that platelets contain both VEGF (Mohle et al., *Proc Natl Acad Sci* 94:663-668 (1997)) and FN (Mosher, *Fibronectin*, xviii, 474 (Academic Press, San Diego, 1989)), experiments were performed to determine whether VEGF/FN complexes exist in platelets. The distribution of VEGF/FN complexes in platelets were examined under both resting and activated conditions as described below.

Washed platelets were prepared as described in Patel et al. (*Thromb Haemost* 79:177-185 (1998)). For activation studies, platelets were resuspended in the presence of 1.5 mM calcium and at a count of 30 $x10^8$/ml. One ml of platelets were stimulated with either saline (resting) or thrombin (1 U/ml) for 10 min. Whole cell lysates were also prepared from unstimulated platelets by lysing them with immunoprecipitation buffer. Supernatants were immunoprecipitated with an antibody to FN (Chemicon International Inc., Temecula, CA). Following SDS-PAGE and immunoblotting, VEGF was detected with a polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) by chemiluminescence.

Immunocytochemical labelling of platelets.

Washed human platelets treated with 1 μM PGE_1 were fixed on slides with 4% (v/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min and permeabilised with 0.01% (v/v) triton X-100 for 5 min. Platelets were incubated with antibodies to FN (Chemicon) and VEGF. For positive identification of α-granules, an antibody to P-selectin (CD62P, Chemicon International Inc.) was used. Secondary antibodies used were goat anti-rabbit or goat anti-mouse IgG conjugated

to fluorescein or Cy3 respectively (Chemicon). Labelled platelets were then mounted in fluoromount (BDH) or PBS/glycerol and visualised using a Nikon UFX-DX confocal microscope.

Results

Confocal microscopic analysis of resting platelets demonstrated that both VEGF and FN co-localised in the α-granules as confirmed by P-selectin staining (data not shown). To test whether VEGF/FN complexes were pre-formed or formed only following platelet activation, whole cell lysates and supernatants of resting and activated platelets were examined for the presence of VEGF/FN complexes. Immunoprecipitation of supernatants from activated platelets with antibodies to FN resulted in significant co-precipitation of VEGF compared to resting platelets (data not shown). A minor amount of co-precipitation of VEGF from the resting supernatant was observed which was due to low level platelet activation during the isolation procedure. However, in whole cell lysates, VEGF was not immunoprecipitated with an antibody to FN. These results indicate that platelet activation is necessary for VEGF/FN complex formation and demonstrate that activated platelets are a potential source of VEGF/FN complexes.

Example 7

Identifying the Vitronectin Domain that Mediates Binding to PDGF-BB

The T7Select™ Phage System (from Novagen Inc., Madison, WI) is used to determine the minimal VN amino acid sequence that will support high affinity PDGF-BB binding. The T7Select™ Phage System is a clone selection technique in which a foreign peptide is expressed as a fusion with a bacteriophage coat protein. Peptides up to 50 amino acids long are displayed in high copy numbers on the surface of the phage (about 400 polypeptides per phage). A biopanning procedure using microtiter plates coated with PDGF-BB is used to select phage displaying the VN peptide that binds to PDGF-BB. Phage binding to PDGF-BB are eluted, amplified, and taken through additional cycles and amplification to successively enrich the pool of phage in favor of high affinity binding to PDGF-BB. After 3-4 rounds of biopanning, individual clones are characterized by DNA sequencing. A series of deletions is then created in the VN peptide to determine the minimum DNA sequence that will support PDGF-BB binding. Each deletion is tested for the capacity to bind to PDGF-BB using a slot-blot and solid phase assay methods described in more detail below.

Phage display: The T7Select™ System can be used to determine the PDGF-BB binding site on VN. This method is described in more detail in the

Novagen, Inc., T7Select™ System Manual which is herein incorporated by reference. Briefly, this system consist of three parts: 1) Cloning of peptide coding sequences into T7 vectors, packaging the recombinant molecules into phage, and amplifying the phage to prepare for biopanning; 2) Performing several rounds of biopanning and then amplifying the phage that bind to the target PDGF-BB; 3) Characterization of phage that bind PDGF-BB by DNA sequencing.

The VN cDNA is well characterized and numerous binding sites for a number of molecules have been determined. The DNA sequences coding for between 20-45 amino acids over the entire VN cDNA sequence are amplified using PCR primers. The 20-45 amino acid regions are amplified such that each peptide coding region overlaps with adjacently amplified coding regions. PCR primer pairs used for the DNA amplification of each 20-45 peptide region of VN are designed such that the 5' end of one PCR primer has an EcoRI restriction endonuclease site, while the other PCR primer has an Hind III restriction endonuclease site at its 5' terminus. Following PCR amplification, DNA fragments are digested with EcoRI and Hind III and then covalently joined to the EcoRI and Hind III arms of the T7 bacteriophage vector using DNA ligase. Ligation reactions are added to T7 packaging extracts for *in vitro* packaging. An aliquot of *in vitro* packaged phage is diluted and plated onto bacteria to determine the number of recombinants generated. The recombinants are then amplified by preparing an agar plate bacteriophage lysate and the resulting phage screened by biopanning. ELISA plates, which can by purchased from Novagen, Inc., as part of a T7Select™ Biopanning Kit, are coated with 100 µl of PDGF-BB (1µg/ml) in TBS at 4°C overnight. After washing with TBS and blocking with 1% BSA/TBS, 10^8 phage are screening for PDGF-binding. Following washing with TBS, phage that have bound to the PDGF-BB immobilized to the plastic surface of the ELISA plate well are eluted with 5M NaCl and subjected to an additional round of phage amplification via preparation of a bacterial lysate. The biopanning screen using the PDGF-BB bound to ELISA plates is repeated three times to enrich for T7 phage that display VN protein domains that bind with a high affinity to PDGF-BB. On the third screening, the PDGF-BB binding phage are washed and directly amplified, *in situ*. The coding region of phage recombinant DNA that encodes the PDGF-BB binding domain is then amplified using PCR primers surrounding the multiple cloning site of the T7 phage vector (purchased from Novagen, Inc.). After determining the PDGF-BB binding sequence, peptides are custom made and tested for PDGF-BB binding capacity using the slot blot and solid phase binding assays described below.

Slot-blot assay: Slot-blot binding assays are performed using nitrocellulose membranes (Life Technologies, Grand Island, NY) pre-wetted in PBS in a slot blot apparatus (Hoeffer Scientific Instruments, Piscataway, NJ) under vacuum. 500 ng of the test VN peptide is made up in PBS to a final volume of 250 ml then applied to the membrane under vacuum. After application, the membrane is rinsed with distilled water. Following washing, the membranes are incubated with 50 ml of a 2% BSA/PBS solution at 37°C for one hour with shaking. The nitrocellulose membranes are then incubated with PDGF BB (20 ng/ml) in PBS containing 0.1% BSA at 37°C for one hour with shaking. After three quick 50 ml PBS washes, the membrane is incubated with a monoclonal antibody to PDGF-BB (from R & D System, Minneapolis, MN) in 1% BSA/PBS for 30 minutes at 37°C with shaking followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories, Inc., Hercules, CA) in 1% BSA/TBS for 20 minutes at 37°C with shaking. Following washing, the nitrocellulose membrane is developed using Super Signal chemiluminescent reagent (Bio-Rad Laboratories, Inc.). Control peptides consist of non-binding PDGF-BB peptides.

Solid-phase binding assay: Microtiter plates are coated with PDGF-BB binding peptides (50 µl; 1 µg/ml) in 0.1 M bicarbonate buffer (pH 9) overnight at 4°C. After washing with PBS, the microtitre plates is blocked with 20 mM Hepes (pH 7.4) containing 0.137 mM NaCl, 1 mM $MgCl_2$ and 0.1% BSA (binding buffer) at 37°C for 30 minutes. 2 ng of ^{125}I-PDGF-BB (NEN Life Science, Boston, MA) in binding buffer containing 1 µg of unlabeled PDGF is added to the microtitre plates and incubated for one hour at room temperature. After washing the plates, they are incubated with 0.1M NaOH for 30 minutes and the amount of bound ^{125}I-PDGF-BB determined by using a gamma counter. The specificity of binding is determined by performing control experiments with 2 ng of ^{125}I-PDGF-BB in a binding buffer containing 500 ng of unlabeled PDGF-BB.

The peptides containing a PDGF-BB binding domain are tested for their ability to inhibit the migration-enhancing activity exhibited by the vitronectin/PDGF complexes in the Transwell assay. The cell migration assay of Example 2 is used to perform experiment in which varying amounts of the VN-PDGF-BB binding domain peptide is mixed with vitronectin in the microwells prior to addition of PDGF-BB. By preventing the formation of vitronectin/PDGF complexes, the binding domain peptide abolishes in a dose-dependent manner the enhanced cell migration seen in the Transwell experiments of Example 2.

Example 8

Fibronectin and Vitronectin Fragments that Inhibit VEGF and/or PDGF-BB Binding to Cells

To test whether fragments of the fibronectin protein are capable of physiologically inhibiting the cell migration inducing activity of VEGF and PDGF the following assay is performed. Human microvessel endothelial cells are cultured in 24-well plates until confluent in MCDB 131 medium containing 5% FBS. After washing the cells twice with PBS, the cells are incubated in sodium bicarbonate free-MCDB 131 containing 10 mM Hepes buffer and 0.5% BSA with 1 ng of ^{125}I-VEGF or ^{125}I-PDGF-BB (purchased from NEN Life Science, Boston, MA) in the presence and absence of the fibronectin or vitronectin fragments to be tested for inhibition of VEGF or PDGF-BB binding to cells. The binding assay sample is incubated for one hour on ice. The cells are then washed with PBS to remove unbound ^{125}I-growth factor. The cells are then treated with 0.1 M sodium hydroxide and the amount of radioactive growth factor bound to the cells determined by use of a gamma radiation counter. FN and VN fragments that prevent the binding of VEGF or PDGF, respectively, to microvessel endothelial cells are suitable as a cell migration inhibitory substance.

Example 9

Fibronectin Fragments that Inhibit VEGF Signaling in Endothelial Cells

In order to assess the significance of the amino terminal FN and carboxy terminal VEGF complexes, studies were performed to measure the effects of these VEGF/FN complexes on endothelial cell migration and differentiation.

Cell Migration

FIGURE 6A shows the effects of VEGF/ECM protein complexes on endothelial cell migration. HMVEC cells plated onto vitronectin coated transwells were exposed to BSA, VEGF, or VEGF with ECM proteins or FN peptides. The number migrating cells were terminal after 6 hours. Endothelial cells that were exposed to VEGF/FN complexes exhibited a synergistic effect on cell migration. The VEGF/FN complexes increasing cell migration by more than 2.5 fold compared to VEGF/collagen I or VEGF/vitronectin combinations (FIGURE 6A). However, when endothelial cells were exposed to VEGF and the FN 120 kDa peptide, endothelial migration was similar to that observed with VEGF alone. Addition of both the FN amino terminal 70 kDa peptide and the FN carboxy terminal 40 kDa peptide to the VEGF/120 kDa mixture failed to restore endothelial migration to levels observed with the VEGF/FN combination (FIGURE 6A). These observations

show that the synergistic effect of the VEGF/FN complex on endothelial cell migration requires FN molecules containing both the amino and carboxy terminal VEGF binding domains.

Cell Differentiation

The effect of the VEGF/FN complex on the differentiation of $CD34^+$ hematopoietic cells to endothelial cells was also examined. $CD34^+$ cell culture was carried out as described previously with minor modifications (Shi et al., *Blood* **92**:362-367 (1998)). Briefly, 6-well plates were coated with FN, collagen I or vitronectin (10 μg/ml) overnight at 4 °C. VEGF (20 ng/ml) was added to the extracellular matrix protein coated plates and incubated at 37 °C for a further hour. Human foetal liver $CD34^+$ cells (Poietic Technologies Inc., Gaithersburg, MD) were then seeded ($1x10^4$ cells) in MCDB-131 medium supplemented with VEGF (10 ng/ml), bFGF (1 ng/ml), IGF-1 (1 ng/ml), Flt-3 ligand (10 ng/ml) and 5% foetal bovine serum. Endothelial cell colonies were identified by immunostaining for von Willebrand Factor (DAKO Corp., Carpinteria, CA) and CD31 (Pharmigen, San Diego, CA).

As shown in FIGURE 6B, endothelial cell colonies derived from $CD34^+$ cell differentiation were observed for all the VEGF/extra cellular matrix protein combinations tested. When $CD34^+$ cells were incubated on VEGF/FN coated plates, there was more than a 5-fold increase in endothelial colonies, indicating that FN was the preferred extra cellular matrix protein. Plates coated with the VEGF/120 kDa peptide and VEGF were not as effective as the intact FN molecule plus VEGF at stimulating the differention of $CD34^+$ cells into endothelial cells. Adding both the amino terminal 70 kDa and carboxy terminal 40 kDa peptides to the VEGF/120 kDa peptide coated plates also failed to restore endothelial cell differentiation to the levels observed with intact FN. These findings show that an intact FN molecule is required to obtain a maximal VEGF stimulation of $CD34^+$ differentiation into endothelial cells. Interestingly, VEGF/fibrinogen coated plates also stimulated endothelial colony formation, indicating an important role for fibrinogen in promoting $CD34^+$ differentiation into endothelial cells during wound healing (FIGURE 6B).

Example 10

Effects of VEGF/FN Complex On Integrin/VEGF Receptor Interaction

The mechanism through which VEGF binding domains of FN enhanced the effects of VEGF on endothelial cell migration and differentiation was studied by first determining whether VEGF/FN complexs promote the association of VEGF receptor FEGFR-1 with various integrin molecules.

Human microvessel endothelial cells (HMVEC) in serum-free MCDB131 media (Clonetics) supplemented with 0.1% BSA were plated on FN, collagen or 120 kDa internal cell binding FN fragment coated plates containing VEGF (20 ng/ml) for 1 h. Cells were lysed with lysis buffer (20 mM HEPES, pH 7.5, containing150 mM n-hexyl-β-D-glucopyranoside, 0.5% Brij 35, 0.2% NP-40, 100 mM NaCl, 5% glycerol, 0.1% BSA and protease inhibitors) and immunoprecipitated with antibodies to α5β1, αv or α2β1 integrin (Chemicon). After SDS-PAGE and immunoblotting, membranes were probed with antibodies to VEGFR-1 (Santa Cruz Biotechnology Inc.) or VEGFR-2 (R&D Systems, Minneapolis, MN). Positive protein bands were visualised by chemiluminescence.

Only cell lysates prepared from endothelial cells incubated on VEGF/FN coated plates exhibited an association of VEGFR-1 with the FN integrin, $\alpha_5\beta_1$ (data not shown). When endothelial cells were incubated on VEGF/vitronectin or VEGF/collagen coated plates, VEGFR-1 could not be immunoprecipitated with antibodies to α_v or $\alpha_2\beta_1$ indicating that VEGFR-1 did not associate with vitronectin receptors ($\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$) or the collagen receptor (data not shown). FLK-1 association with $\alpha_5\beta_1$ integrin was minimal when endothelial cells were incubated on plates coated with VEGF and the FN 120 kDa peptide. Adding the amino terminal FN 70 kDa and carboxy terminal 40 kDa peptides to the VEGF/120 kDa coated plates failed to promote association of VEGFR-1 and $\alpha_5\beta_1$ (data not shown). These observations indicate that both of the VEGF binding domains on FN play a role in promoting the association of VEGFR-1 and $\alpha_5\beta_1$. However, it is important to note that neither the amino terminal FN 70 kDa or the carboxy terminal FN 40 kDa peptides have a RGD integrin binding domain sequence within them. The data also suggests a possible mechanism involving growth factor binding domains in promoting integrin/growth factor receptor association and activation of the signalling pathway mediated by the formation of the $\alpha_5\beta_1$/VEGFR-1 complex. In contrast to VEGFR-1, VEGFR-2 did not associate with the $\alpha_5\beta_1$ integrin (data not shown).

The significance of the dual VEGF binding domains in FN in enhancing the signaling pathway induced by VEGF was further demonstrated by performing experiments to determine if VEGF/FN complexes activate mitogen-activated protein kinase (MAPK). MAPK activation was determined by lysing HMVEC cells treated with various combinations of VEGF/FN complexes and the MAPK protein was immunoprecipitated using a pan MAPK antibody (BD Transduction Laboratories, Lexington, KY). MAPK-immune complex was subjected to an *in vitro* kinase assay as previously described (Kazlauskas et al., *Cell* 58:1121-1133 (1989)).

FIGURE 7 shows that when endothelial cells were plated on VEGF/FN complexes, but not with VEGF/vitronectin or VEGF/120 kDa peptide, a sustained activation of MAPK kinase activity was observed. HMVEC lysates were assayed at the time points indicated in FIGURE 7 for MAPK activity. The data presented in FIGURE 7 demonstrate that an intact FN molecule is required to mediate the VEGF-induced VEGFR-1/$\alpha_5\beta_1$ association and its subsequent prolonged activation of MAPK.

Example 11

VEGF Binding Domain Fragments Inhibit VEGF Activity

Recent studies have demonstrated that proteolytic fragments of extracellular matric proteins and blood-derived proteins such as endostatin and angiostatin can inhibit endothelial cell migration (Ji et al., *Faseb. J.* **12**:1731-1738 (1998); Yamaguchi et al., *EMBO J.* **18**:4414-4423 (1999)). Therefore, the effects of the amino terminal FN 70 kDa and the carboxy terminal 40 kDa peptides on VEGF stimulated migration of endothelial cells were tested using the methods of Example 2. Endothelial cells were exposed to VEGF (20 ng/ml) for 6 hours in the presence or absence of the carboxy terminal 40 kDa and amino terminal 70 kDa FN peptide fragments.

The carboxy terminal FN 40 kDa fragment was found to inhibit VEGF-induced cell migration by over 75% (FIGURE 8A). Similarly, the carboxy terminal FN 40 kDa peptide also had an inhibitory effect (27%) on $CD34^+$ cell differentiation (data not shown). No significant effects were observed with the amino terminal FN 70 kDa fragment (FIGURE 8A).

A possible mechanism through which the carboxy terminal FN 40 kDa fragment exerts its inhibitory effects is that the carboxy terminal FN 40 kDa peptide might be competing with VEGF for binding to FLK-1. FIGURE 8B shows that FN 70 kDa and 40 kDa peptides are capable of inhibiting the binding of ^{125}I-VEGF to VEGFR-1. Recombinant VEGFR-1 immobilized on microtiter plates were incubated with ^{125}I-VEGF and increasing concentrations of the 70 kDa and 40 kDa FN fragments. As shown in FIGURE 8B, ^{125}I-VEGF binding to recombinant VEGFR-1 was inhibited by nearly 80%, whereas the amino terminal FN 70 kDa fragment inhibited ^{125}I-VEGF binding by only 20%.

Direct binding of the carboxy terminal FN 40 kDa fragment to VEGFR-1 was demonstrated by measuring the amount of various FN fragments that bound to recombinant VEGFR-1 immobilized onto nitrocellulose membranes (data not shown). In contrast, the amino terminal FN 70 kDa and middle FN 120 kDa

fragments did not bind to VEGFR-1 immobilized onto nitrocellulose membranes (data not shown).

These data support the conclusion that the inhibitory effect of the carboxy terminal FN 40 kDa peptide on cell migration is mediated by the binding of the FN 40 kDa peptide to VEGFR-1 thereby inhibiting VEGF binding and receptor activation. In contrast, the inhibition of cell migration observer upon treatment of the cells with amino terminal FN 70 kDa peptide is likely to occur through sequestration of VEGF. To confirm this hypothesis, studies were performed to measure the ability of the amino terminal FN 70 kDa and carboxy terminal FN 40 kDa peptides to stimulate MAPK activation and inhibit VEGFR-1 phosphorylation on HMVEC.

VEGFR-1 phosphorylation experiments were performed by exposing HMVEC cells to VEGF (20ng/ml) with or without the 70 kDa and 40 kDa FN peptides (100 ng/ml) for 5 minutes. Cell lysates were then immuno precipitated with anti-phosphotyrosine antibodies followed by immunoblotting with monoclonal VEGFR-1 antibody. Phosphorylation of VEGFR-1 was greatly reduced when endothelial cells were stimulated with VEGF in the presence of the carboxy terminal FN 40 kDa peptide. In contrast, phosphorylation of VEGFR-1 was only slightly decreased when treated with VEGF and the amino terminal 70 kDa peptide (data not shown). Consistent with the decrease in phosphorylated VEGFR-1, the activity of MAPK in endothelial cells was also inhibited by the carboxy terminal FN 40 kDa peptide while the FN 70 kDa peptide showed only a slight decrease compared to cells treated with VEFG alone (data not shown). The amino terminal FN 70 kDa peptide had only a modest effect. Collectively, these findings show that the amino terminal 40 kDa peptide when cleaved from the native FN molecule can function as a potent inhibitor of biological responses induced by VEGF signal transduction. In addition, the carboxy terminal 70 kDa peptides can also act as an inhibitor of the biological responses induced by VEGF, but to a lesser extent than the 40 kDa FN peptides.

Example 12

Fibrin Glue Preparations Containing Fibronectin and VEGF

Fibrin glue preparations were made by first mixing 15 mg/ml fibrinogen with 0.5 mg/ml fibronectin and 8 mg/ml calcium chloride. To initiate gel formation 20 units of factor XIII and 50 units of thrombin were mixed into the first mixture. A wide range of concentrations for each of the components listed above may be used to make a fibrin glue composition. For example, fibrinogen may be used in a range of 10 to 150 mg/ml, fibronectin in a range of 0.25 to 100 mg/ml and thrombin in a range of 20 to 500 units. The biologically active molecules of the present invention may be

added to the above fibrin glue mixture in a concentration range of 50 ng/ml to 1 mg/ml prior to addition of the gel initiation components.

To test the effectiveness of fibrin glue compositions containing fibronectin and VEGF the cell migration assay described in Example 2 was used with the following modifications. MVECs were labeled with ^{3}H-thymidine for 24 hours prior to assay. The Transwells were pre-coated on the underside with fibrin glue compositions as described above. The cell migration assays were performed for 24 hours in the presence of hydroxy urea to block cell proliferation but allow cell migration. After 24 hours of incubation each fiberin glue sample was removed and solubilized in 0.1 M NaOH. The amount of cell migration and adhesion was quantified by measuring the amount of labeled ^{3}H-thymidine present within each solubilized glue sample in a scintillation counter.

The bar graph represented in FIGURE 9, graphically illustrates measurements of MVEC migration in the presence of various fibrin glue compositions with or without fibronectin and VEGF. The brackets at the top of each bar in the graph shown in FIGURE 9 represent one standard deviation. The results show that the fiberin glue composition containing both fibronectin and VEGF (bar C) promotes greater cell migration then do glue compositions that contain no fibronectin or VEGF (bar A) or contain only VEGF (bar B).

Example 13

Construction of Chimeric Proteins

The chimeric proteins of the present invention can be constructed using the polymerase chain reaction (PCR) based methods as described by Higuchi (*PCR Technology: Principles and Applications for DNA Amplification*, Stockton Press, New York, p. 61-70 (1989)) and Pont Kingdom (*Biotechniques*, **16**:1010-1011 (1994)). In the specific embodiments described below reference to protein domains capable of binding to an integrin receptor should be understood to include a RGD amino acid sequences as well as the amino acid residues flanking this site that help determine whether a ligand containing a RGD integrin binding site will bind to bind to $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_5\beta_1$ and or any other integrin that will promote angiogenesis.

Chimera consisting of fibronectin (FN) VEGF-binding fragment linked to a RGD sequence

It is well known in the art that a wide variety of PCR based techniques can be used to add specific nucleic acid sequences to either end of a larger DNA molecule. To make chimeric proteins containing both a FN VEGF-binding fragment and an integrin receptor binding domain, the following PCR method can be used. PCR

primers A and B are used to amplify a FN VEGF-binding fragment molecule that contains unique restriction endonuclease enzyme sites at both ends, and in addition, contains the coding region for an integrin receptor binding domain fused in frame with the FN VEGF-binding fragment.

The nucleotide sequence at the 3' end of primer A is selected such that it contains a sequence that is identical to a 15 to 30 base sequence located at the 5' end of the FN VEGF-binding fragment coding strand (*See* Kornblihtt et al., *Proc. Natl. Acad. Sci. U.S.A.* **80**:32118-3222 (1983)) for the complete nucleotide sequence of fibronectin). Primer A is complementary to sequences on the template strand of the FN VEGF-binding fragment. The 5' end of primer A is selected such that it encodes a restriction endonuclease that is unique to the PCR amplified FN VEGF-binding fragment DNA molecule. This restriction enzyme site is useful for the directional cloning of the amplified fragment into a plasmid or viral vector suitable for expressing the FN VEGF-binding fragment/integrin binding domain fusion protein within the desired host organism. Suitable protein expression vectors are well known in the art of molecular biology as reflected in Hitzeman et al. (U.S. Patent No. 5,618,676) and references cited therein.

Practitioners of the art of genetic engineering well appreciate that many different possible PCR primer sequences can be designed having the characteristics delineated for primer A. One possible example of primer A which will function to accomplish the construction of the inventive chimeric proteins is: 5'-CAG GCT CAG CAA ATG GTT CA- 3' [SEQ ID 1].

Primer B is selected such that it is complementary at its 3' end to a 20 to 60 nucleotide sequence located at the 3' end of the FN VEGF-binding fragment coding strand. The middle region of primer B contains nucleotide bases selected to encode the amino acid sequence for RGD. Lastly, the 5' end of primer B contains a restriction endonuclease site that is unique to the final PCR amplification product. Again, this added restriction enzyme site aids in the subsequent cloning of the amplified DNA fragment into a suitable expression vector. One possible embodiment of primer B is: 5'-TGA GCT TGG ATA GGT CTG TGT TCA CTG AGC GCC CCT ACA CAA GTG ATA C- 3'[SEQ ID 2].

Chimera consisting of FN VEGF binding fragment and vitronectin (VN) PDGF-binding fragment linked by a RGD integrin receptor binding domain

To make chimeric proteins containing a FN VEGF-binding fragment and a VN PDGF-binding fragment both fused to a peptide sequence capable of binding to an integrin receptor the following PCR method can be used. In a first round of PCR,

two different DNA products are independently amplified from two different DNA molecules containing either the coding region for the FN VEGF-binding fragment or the VN PDGF-binding fragment. The first product is amplified with PCR primers C and D that are complementary to nucleotide sequences flanking the coding region of the FN VEGF-binding fragment.

The nucleotide sequence of the 3' end of primer C is selected such that it is identical in sequence to a 15 to 20 nucleotide sequence located at the 5' end of the FN VEGF-binding fragment coding strand. The 5' end of primer C is selected such that it encodes a restriction endonuclease that is unique to the PCR amplified FN VEGF-binding fragment DNA molecule. This restriction enzyme site is useful for the directional cloning of the amplified fragment into a plasmid or viral vector suitable for expressing the FN VEGF-binding fragment/RGD integrin binding domain fusion protein within the desired host organism. The nucleotide sequence represented as SEQ ID 1 is one possible embodiment of primer C.

The nucleotide sequence of primer D is selected such that it is complementary at its 31' end to a 15 to 20 nucleotide sequence located at the 3' end of the FN VEGF-binding fragment coding strand. The middle region of primer D contains nucleotide bases selected to encode a RGD amino acid sequence. The 5' end of primer D contains a 10 to 20 nucleotide sequence that is complementary to the 5' end of primer E (primer E is described below). PCR products of this first amplification have a FN VEGF binding fragment coding region fused in proper reading frame to a peptide sequence capable of binding to an integrin receptor. One possible embodiment of Primer D is: 5' -TCA TAG TGA ACA CAT CCC CGC GAG TCA CTT GTG AGC TTG GAT AGG TCT GT- 3' [SEQ ID 3].

Primers E and F are used to amplify a second PCR product from the coding region of a VN PDGF-binding fragment coding sequence (*see* Jenne et al., *EMBO J.* 4:3153-3157 (1985)) for the complete nucleotide sequence of vitronectin). The 5' terminal sequence of primer E contains 15 to 30 nucleotides that are complementary to the 5' terminal sequence of primer D and are further selected to maintain an open reading frame with the nucleotides complementary to the N-terminal coding region of the VN PDGF-binding fragment. The nucleotide sequence of the 3' terminus of primer E is selected such that it has 15 to 30 nucleotides that are identical to sequences located at the 5' end of the VN PDGF-binding fragment coding strand. One possible embodiment of primer E is: 5' -CAA GTG ACT CGC GGG GAT GTG TTC ACT ATG ATG GCA CCC CGC CCC TCC TTG AC- 3' [SEQ ID 4].

The nucleotide sequence of primer F is selected such that the bases near its 5' terminus encode a restriction endonuclease enzyme cutting site that is unique within the DNA sequence which encodes the final FN VEGF-binding fragment/integrin receptor binding region/VN PDGF-binding fragment fusion coding sequence. The sequence of the 3' region of primer F is selected such that it is complementary to a 15 to 20 nucleotide sequence located at the 5' end of the VN PDGF-binding fragment coding strand. One possible embodiment of primer F is: 5'-CAG ATG GCC AGG AGC TGG GCA-3' [SEQ ID 5].

After two independent first PCR amplification reactions are carried out with primers C and D, and primers E and F, respectively, the amplification products are denatured and mixed together to perform a second PCR amplification using primers C and F. The resulting target PCR product contains the coding region of the FN VEGF-binding fragment fused in-frame with a RGD integrin receptor-binding domain, which is in turn fused in-frame with the VN PDGF-binding fragment coding sequence. Unique restriction endonuclease sites are additionally contained at either end of the second PCR amplification product that can be digested with appropriate restriction endonuclease enzymes to facilitate the directional cloning of the second amplified DNA fragment into a plasmid or viral vector which is suitable for expressing the FN VEGF-binding fragment/integrin receptor binding domain/VN PDGF-binding fragment fusion protein within the desired host organism.

Chimeric proteins containing multiple copies of the above described fusion proteins may be made using a wide variety of gene cloning techniques that are well known in the art. In addition, artisans will well appreciate the importance of constructing gene fusions encoding these multimer proteins in such fashion as to maintain a proper protein translation reading frame at each of the junctions between the DNA sequences encoding a copy of the fibronectin/integrin receptor binding site fusion protein or fibronectin/ RGD integrin receptor binding site/vitronectin fusion protein.

<u>Chimera consisting of FN VEGF-binding fragment and vitronectin (VN) PDGF-binding fragment</u>

To make chimeric proteins containing both a FN VEGF-binding fragment and a VN PDGF-binding fragment a two step PCR amplification method similar in strategy to that described for the chimeric protein containing a FN VEGF-binding fragment and a VN PDGF-binding fragment both fused to a peptide sequence capable of binding to a RGD integrin receptor is used. In a first round of PCR, two different DNA products are independently amplified from two different DNA

molecules containing either the coding region for the FN VEGF-binding fragment or the VN PDGF-binding fragment. The first product is amplified with PCR primers C and G that are complementary to nucleotide sequences flanking the coding region of the FN VEGF-binding fragment.

The nucleotide sequence of primer G is selected such that it is complementary at its 3' end to a 15 to 20 nucleotide sequence located at the 3' end of the FN VEGF-binding fragment coding strand. The 5' end of primer G contains a 10 to 20 nucleotide sequence that is complementary to the 5' end of primer H (primer H is described below). PCR products of this first amplification have a FN VEGF binding fragment coding region fused in proper reading frame to a short linker peptide sequence. One possible embodiment of primer G is: 5' -TCA TAG TGA ACA CAG TCA CTT GTG AGC TTG GAT AGG TCT GT- 3' [SEQ ID 6].

Primers H and F are used to amplify a second PCR product from the coding region of a VN PDGF-binding fragment coding sequence (*see* Jenne et al., *EMBO J.* 4:3153-3157 (1985)) for the complete nucleotide sequence of vitronectin). The 5' terminal sequence of primer H contains 15 to 30 nucleotides that are complementary to the 5' terminal sequence of primer G and are further selected to maintain an open reading frame with the nucleotides complementary to the N-terminal coding region of the VN PDGF-binding fragment. The nucleotide sequence of the 3' terminus of primer H is selected such that it has 15 to 30 nucleotides that are identical to sequences located at the 5' end of the VN PDGF-binding fragment coding strand. One possible embodiment of primer H is: 5' -CAA GTG ACT GTG TTC ACT ATG ATG GCA CCC CGC CCC TCC TTG AC- 3' [SEQ ID 7].

After two independent first PCR amplification reactions are carried out with primers C and G, and primers H and F, respectively, the amplification products are denatured and mixed together to perform a second PCR amplification using primers C and F. The resulting target PCR product contains the coding region of the FN VEGF-binding fragment fused in-frame with the VN PDGF-binding fragment coding sequence. Unique restriction endonuclease sites are additionally contained at either end of the second PCR amplification product that can be digested with appropriate restriction endonuclease enzymes to facilitate the directional cloning of the second amplified DNA fragment into a plasmid or viral vector which is suitable for expressing the FN VEGF-binding fragment/VN PDGF-binding fragment fusion protein within the desired host organism.

Example 14

Testing the Cell Migration Inducing Efficacy of Chimeric Proteins

The endothelial migration assay described in Example 12 and a chick embryo chorioallantoic membrane (CAM) assay are used to determine the effectiveness of the chimeric proteins in promoting angiogenesis. The CAM model is frequently used to test for angiogenic and anti-angiogenic factors. The assay is well known in the art and is performed as described by Dammacco et al. (*Experimental Hematology*, **26**:1215-1222 (1998)). In addition, the chimeric proteins of the present invention are incorporated into a matrix, such as provided in Example 8, in order to facilitate the therapeutic delivery of these proteins to in vivo sites in need of stimulation or inhibition of endothelial cell migration, angiogenesis and wound healing.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A composition comprising an isolated complex formed between vascular endothelial growth factor and fibronectin.

2. A composition according to Claim 1, further comprising a matrix.

3. A composition according to Claim 2 wherein the matrix is fibrin glue.

4. A composition according to Claim 2 wherein the matrix is a biodegradable polymer selected from the group consisting of hyaluronic acid, chondroitin sulfate, heparin, heparin sulfate, polylactate, polyglycolic acid, starch and collagen.

5. A chimeric polypeptide comprising a peptide fragment of fibronectin that is capable of binding to vascular endothelial growth factor and that is covalently joined to a peptide domain comprising an RGD amino acid sequence that is capable of binding to an integrin receptor.

6. A multimer chimeric polypeptide comprising multiple copies of the chimeric polypeptide of Claim 5.

7. A chimeric polypeptide according to Claim 5 which further comprises a peptide fragment of vitronectin capable of binding to platelet derived growth factor-BB that is covalently joined to the peptide domain comprising an RGD amino acid sequence.

8. A chimeric polypeptide according to Claim 5 wherein the peptide fragment of fibronectin capable of binding to vascular endothelial growth factor is a 70 kDa peptide derived from the amino terminus of fibronectin.

9. A chimeric polypeptide according to Claim 5 wherein the peptide fragment of fibronectin capable of binding to vascular endothelial growth factor is a 40 kDa peptide derived from the carboxy terminus of fibronectin.

10. A composition comprising a complex formed between the chimeric polypeptide of Claim 7, vascular endothelial growth factor and platelet derived growth factor-BB.

11. A composition according to Claim 10, further comprising a matrix.

12. A composition according to Claim 11 wherein the matrix is fibrin glue.

13. A composition according to Claim 11 wherein the matrix is a biodegradable polymer selected from the group consisting of hyaluronic acid, chondroitin sulfate, heparin, heparin sulfate, polylactate, polyglycolic acid, starch and collagen.

14. A multimer comprising multiple copies of a peptide fragment of fibronectin capable of binding to vascular endothelial growth factor covalently joined to a peptide domain comprising an RGD amino acid sequence that is capable of binding to an integrin receptor, the peptide domain being covalently joined to a peptide fragment of vitronectin that is capable of binding to platelet derived growth factor.

15. A chimeric polypeptide comprising a peptide fragment of fibronectin which is capable of binding to vascular endothelial growth factor that is covalently joined at its carboxyl terminus to a peptide fragment of vitronectin which is capable of binding to platelet derived growth factor-BB, said chimeric polypeptide lacking a RGD peptide domain that is capable of binding to an integrin receptor.

16. A chimeric polypeptide according to Claim 15 wherein the peptide fragment of fibronectin is a 70 kDa fragment from the amino terminus.

17. A chimeric polypeptide according to Claim 15 wherein the peptide fragment of fibronectin is a 40 kDa fragment from the carboxy terminus.

18. A protein multimer comprising multiple copies of a peptide fragment of fibronectin which is capable of binding to vascular endothelial growth factor that is covalently joined at its carboxy terminus to a peptide fragment of vitronectin which is capable of binding to platelet derived growth factor-BB, said protein multimer lacking a RGD peptide domain capable of binding to an integrin receptor.

19. A method of promoting angiogenesis comprising contacting cells with an isolated composition comprising a complex formed between vascular endothelial growth factor and fibronectin.

20. A method according to Claim 19 wherein the composition further comprises a matrix.

21. A method according to Claim 20 wherein the matrix is fibrin glue.

22. A method of promoting angiogenesis comprising exposing cells to a composition comprising a complex formed between vascular endothelial growth factor and a chimeric polypeptide, wherein the chimeric polypeptide comprises a peptide fragment of fibronectin that is capable of binding to vascular endothelial growth factor that is covalently joined to a RGD peptide domain that is capable of binding to an integrin receptor.

23. A method according to Claim 22 wherein the peptide fragment of fibronectin is a 70 kDa fragment from the amino terminus.

24. A method according to Claim 22 wherein the peptide fragment of fibronectin is a 40 kDa fragment from the carboxy terminus.

25. A method according to Claim 22 wherein the chimeric polypeptide is a multimer comprising multiple copies of a peptide fragment of fibronectin that is capable of binding to vascular endothelial growth factor.

26. A method according to Claim 22 wherein the composition further comprises a matrix.

27. A method according to Claim 26 wherein the matrix is fibrin glue.

28. A method according to Claim 22 wherein the composition further comprises a complex formed between platelet derived growth factor and the chimeric polypeptide, wherein the chimeric polypeptide further comprises a peptide fragment of vitronectin that is capable of binding to platelet derived growth factor-BB that is covalently to the RGD peptide domain.

29. A method according to Claim 28 wherein the composition further comprises a matrix.

30. A method according to Claim 29 wherein the matrix is fibrin glue.

31. A method of inhibiting a physiological response of a vascular endothelial cell to vascular endothelial growth factor comprising exposing the cell to

a protein fragment from fibronectin that binds to vascular endothelial growth factor and does not contain a RGD peptide domain that is capably of binding to an integrin receptor.

32. A method according to Claim 31 wherein the protein fragment comprises a 70 kDa fragment from the amino terminus.

33. A method according to Claim 31 wherein the protein fragment comprises a 40 kDa fragment from the carboxy terminus.

34. A method according to Claim 31 wherein the physiological response is cell migration.

35. A method according to Claim 31 wherein the physiological response is cell differentiation.

36. A method according to Claim 33 wherein the physiological response is cell migration.

37. A method according to Claim 33 wherein the physiological response is activation of mitogen-activated protein kinase.

38. A method of inhibiting a physiological response of a cell to vascular endothelial growth factor and platelet derived growth factor-BB comprising exposing the cell to a chimeric polypeptide comprising a peptide fragment of fibronectin which is capable of binding to vascular endothelial growth factor that is covalently joined to a peptide fragment of vitronectin which is capable of binding to platelet derived growth factor-BB, said chimeric polypeptide lacking a RGD peptide domain that is capably of binding to an integrin receptor.

39. A method of stimulating the migration of a vascular endothelial cell comprising exposing the cell to a composition comprising an isolated complex formed between vascular endothelial growth factor and fibronectin.

40. A method according to Claim 39 wherein the composition further comprises a matrix.

41. A method according to Claim 39 wherein the matrix is fibrin glue.

42. A method for inhibiting VEGF receptor function comprising:

a. contacting a carboxy terminal fragment of fibronectin with VEGF to form a VEGF/fibronectin fragment complex, wherein the carboxy terminal fragment of fibronectin contains a VEGF binding site and lacks a RGD domain that is capable of binding to a integrin receptor; and

b. contacting the VEGF receptor with the VEGF/fibronectin fragment complex to inhibit VEGF receptor function.

43. A method according to Claim 42 wherein the VEGF-receptor comprises at least one VEGF receptor chosen from VEGFR-1 and VEGFR-2 and the carboxy terminal fragment of fibronectin comprises about 40 kDa.

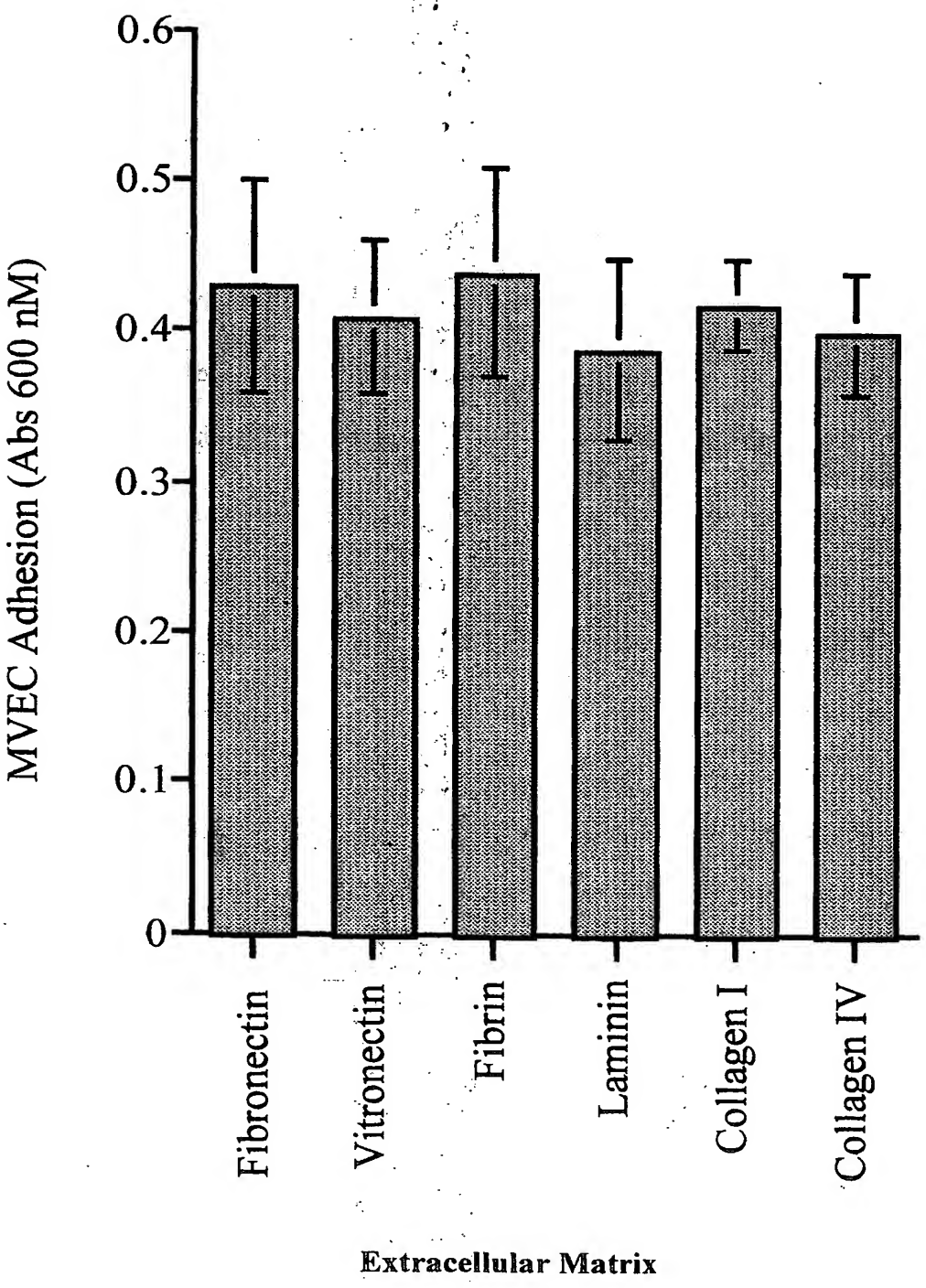
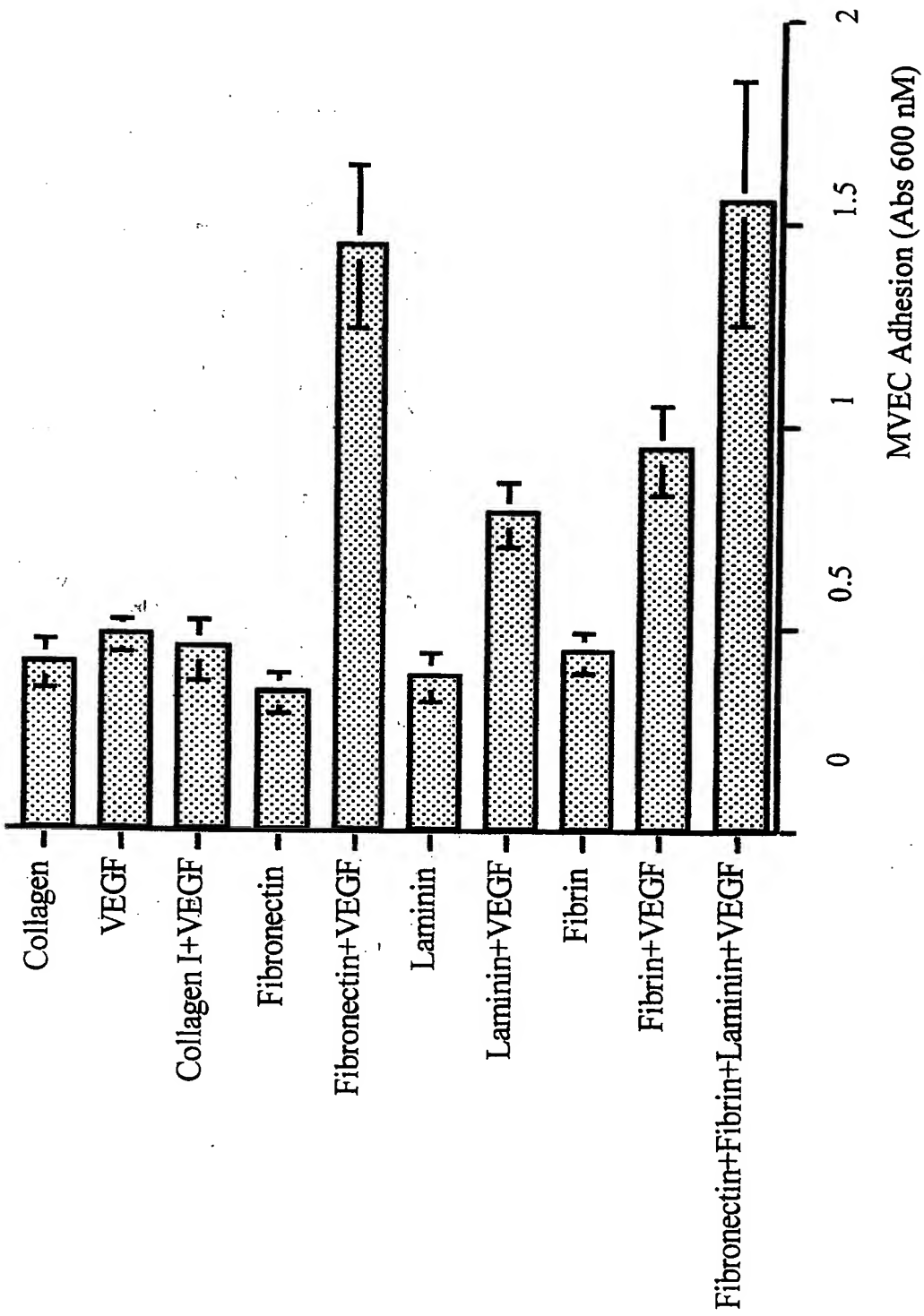

MVEC Adhesion (Abs 600 nM)
0.6
0.5
0.4
0.3
0.2
0.1
0
Fibronectin
Vitronectin
Fibrin
Laminin
Collagen I
Collagen IV
Extracellular Matrix

Fig. 1


Collagen
VEGF
Collagen I+VEGF
Fibronectin
Fibronectin+VEGF
Laminin
Laminin+VEGF
Fibrin
Fibrin+VEGF
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0
0.5
1
1.5
2
MVEC Adhesion (Abs 600 nM)

Fig. 2

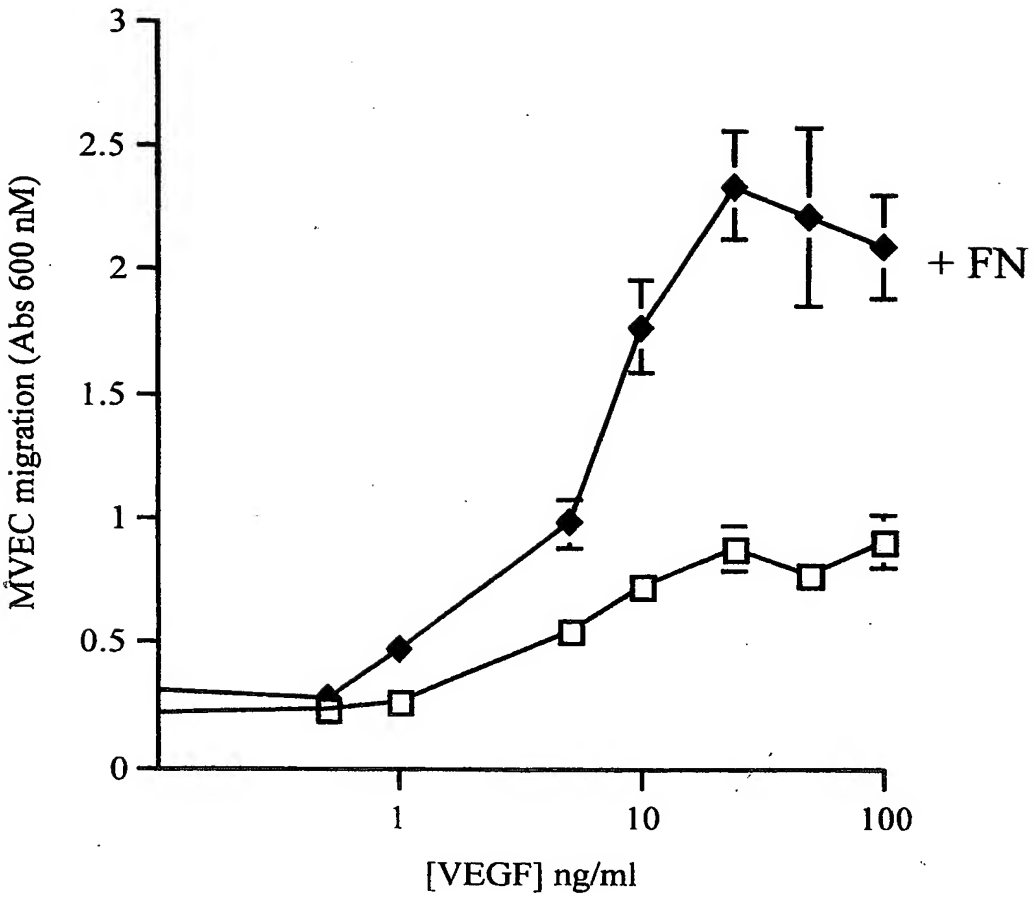


Fig. 3

Fig. 4A

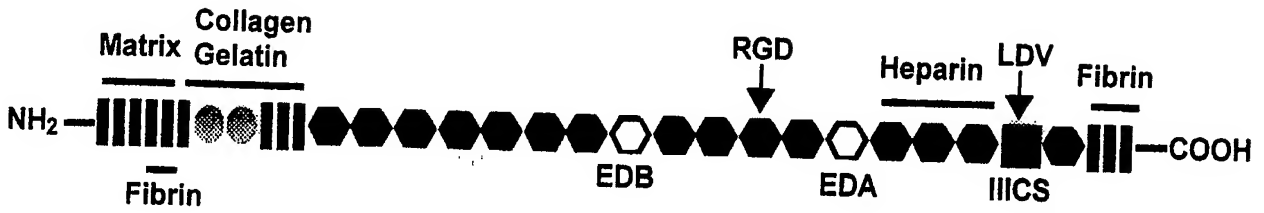
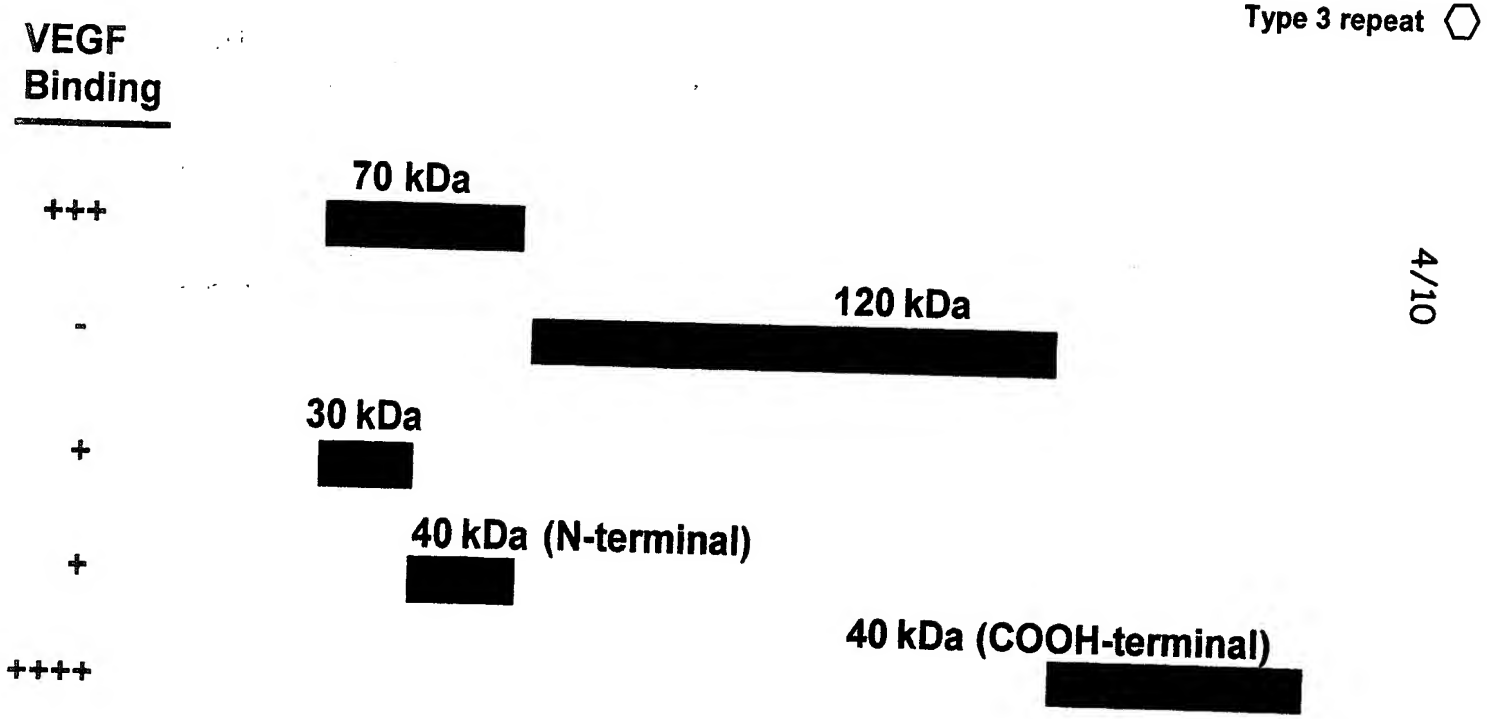


Fig. 4B.



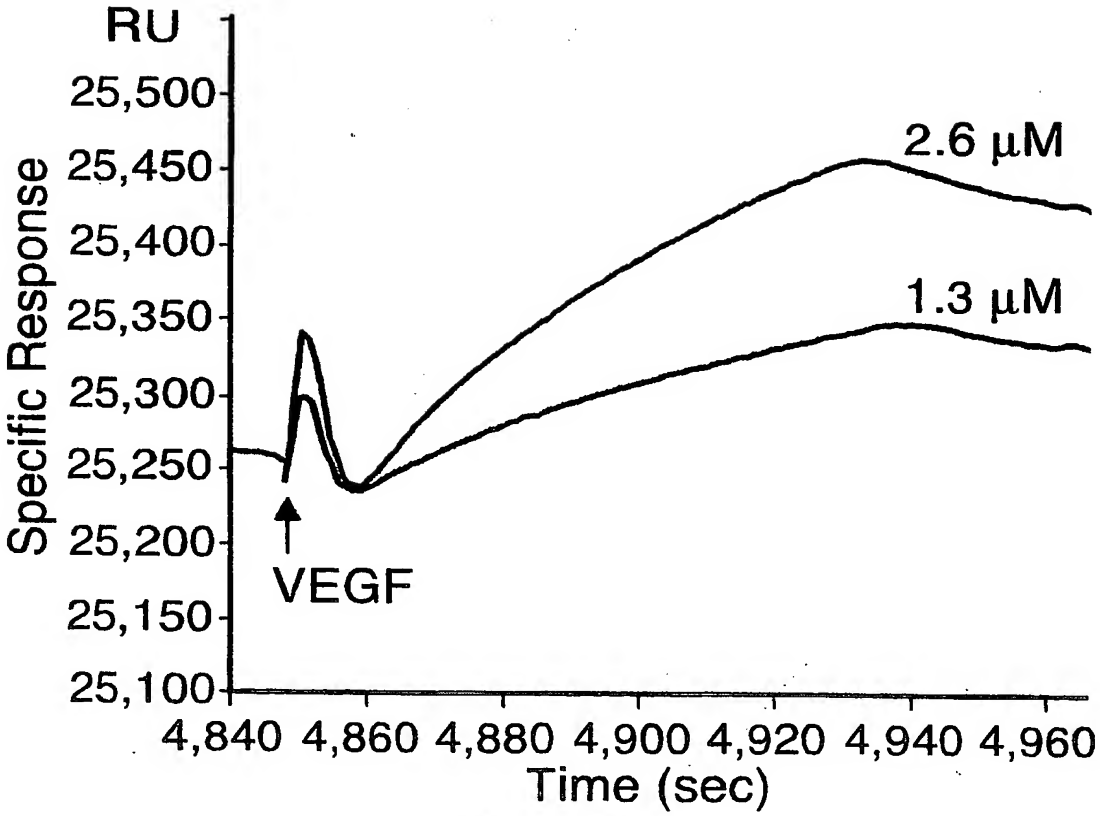

RU
25,500
25,450
25,400
25,350
25,300
25,250
25,200
25,150
25,100
Specific Response
2.6 µM
1.3 µM
VEGF
4,840 4,860 4,880 4,900 4,920 4,940 4,960
Time (sec)

Fig. 5

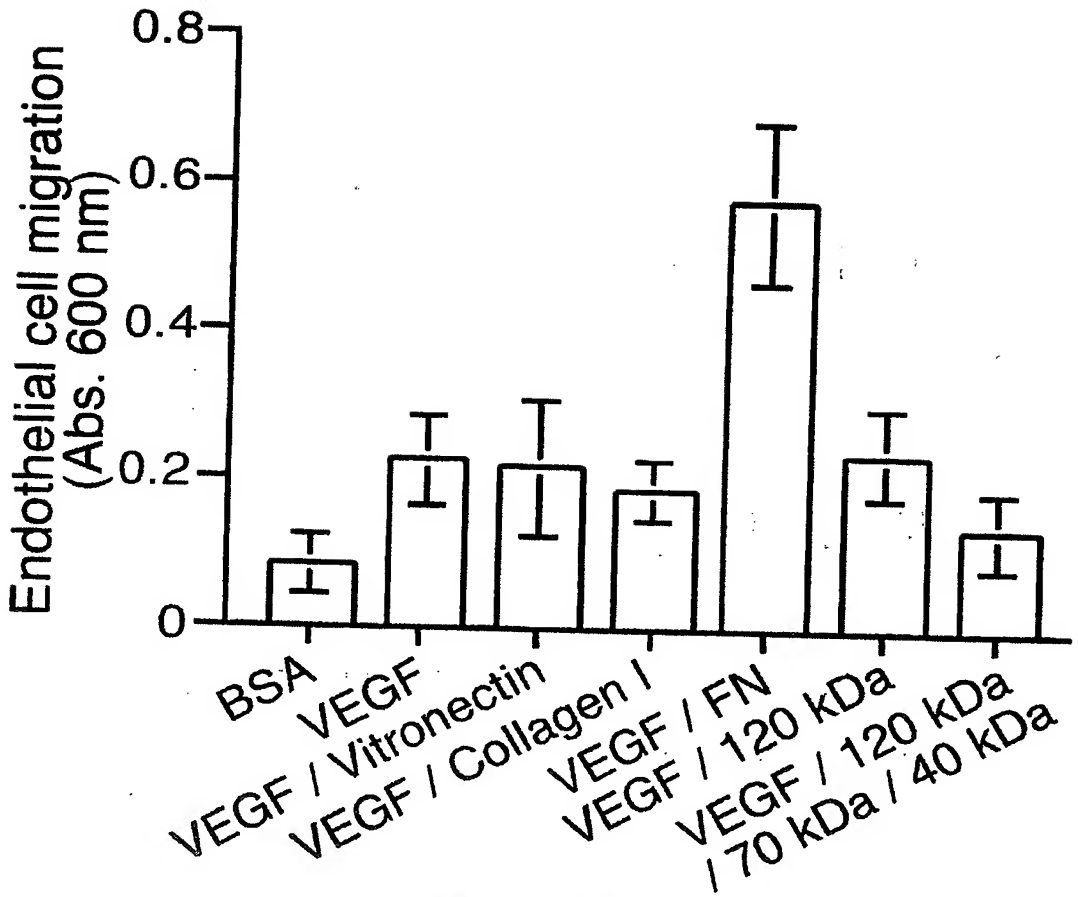

Endothelial cell migration (Abs. 600 nm)
0.8
0.6
0.4
0.2
0
BSA
VEGF
VEGF / Vitronectin
VEGF / Collagen I
VEGF / FN
VEGF / 120 kDa
VEGF / 120 kDa / 70 kDa / 40 kDa

Fig. 6A

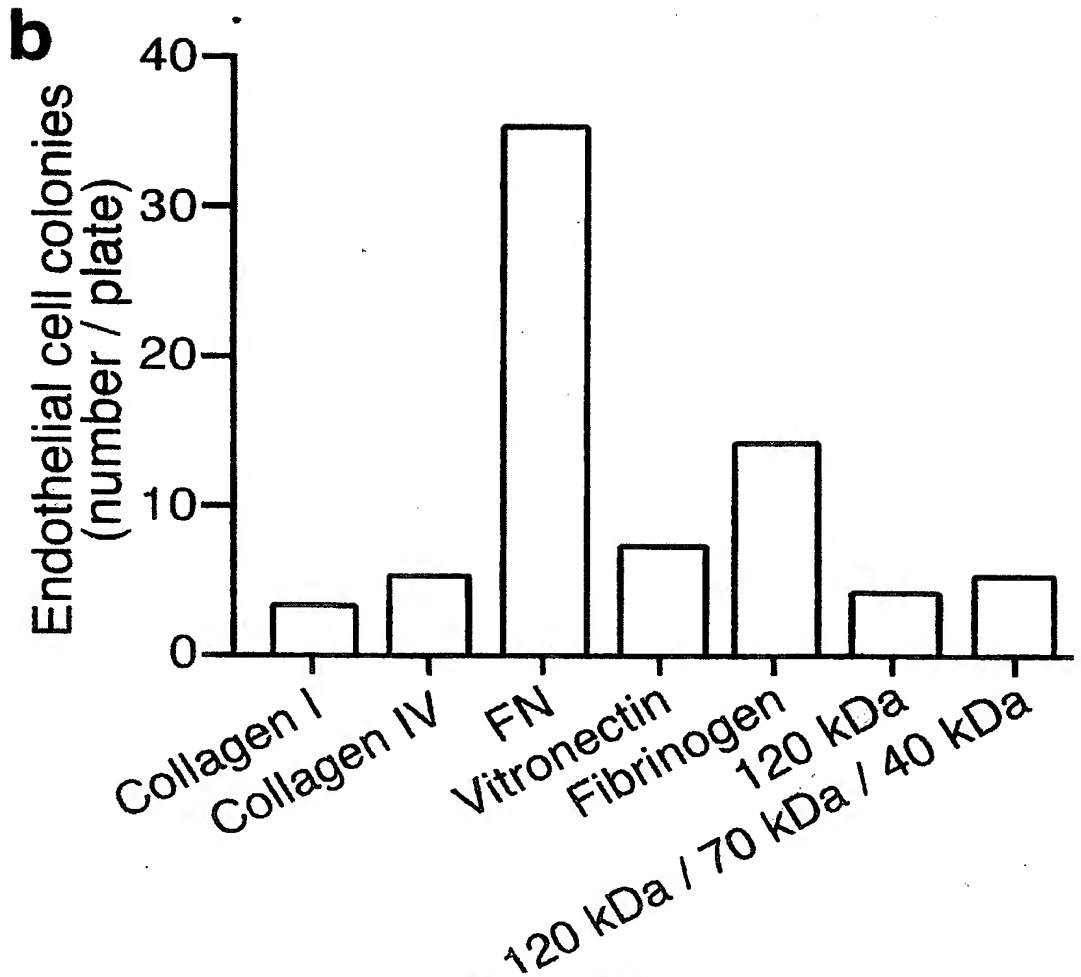
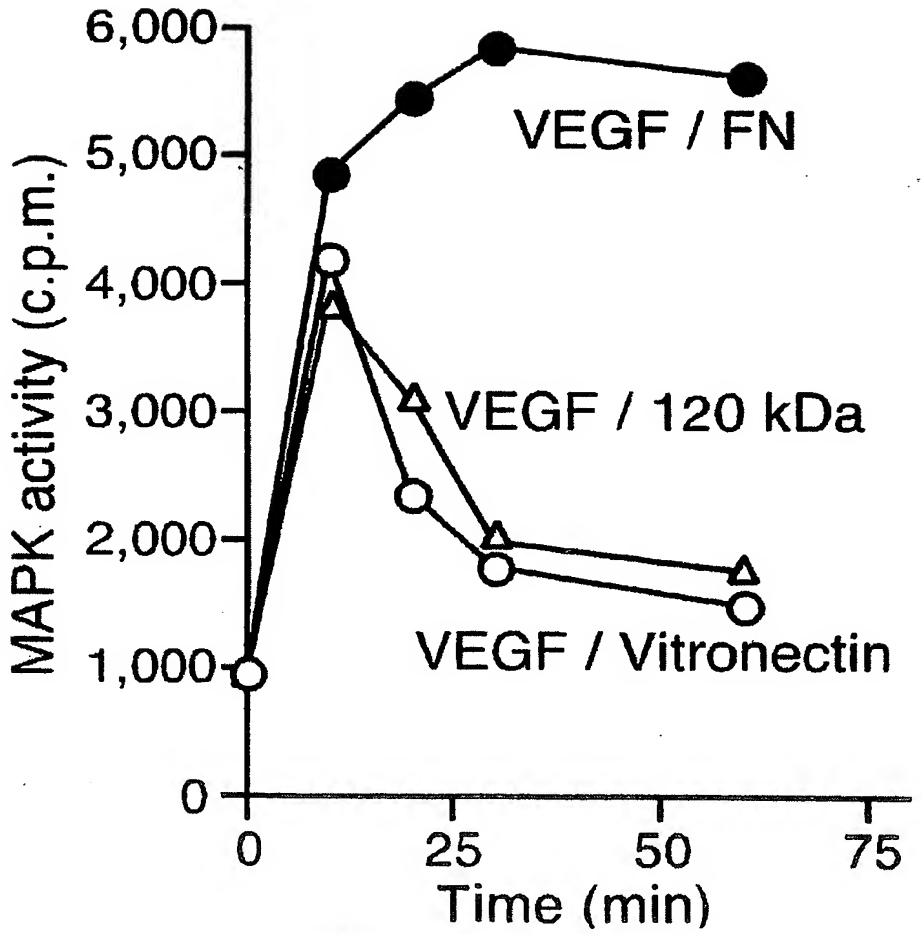

b
Endothelial cell colonies (number / plate)
40
30
20
10
0
Collagen I
Collagen IV
FN
Vitronectin
Fibrinogen
120 kDa
120 kDa / 70 kDa / 40 kDa

Fig. 6B


6,000
5,000
4,000
3,000
2,000
1,000
0
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VEGF / FN
VEGF / 120 kDa
VEGF / Vitronectin
0
25
50
75
Time (min)

Fig. 7

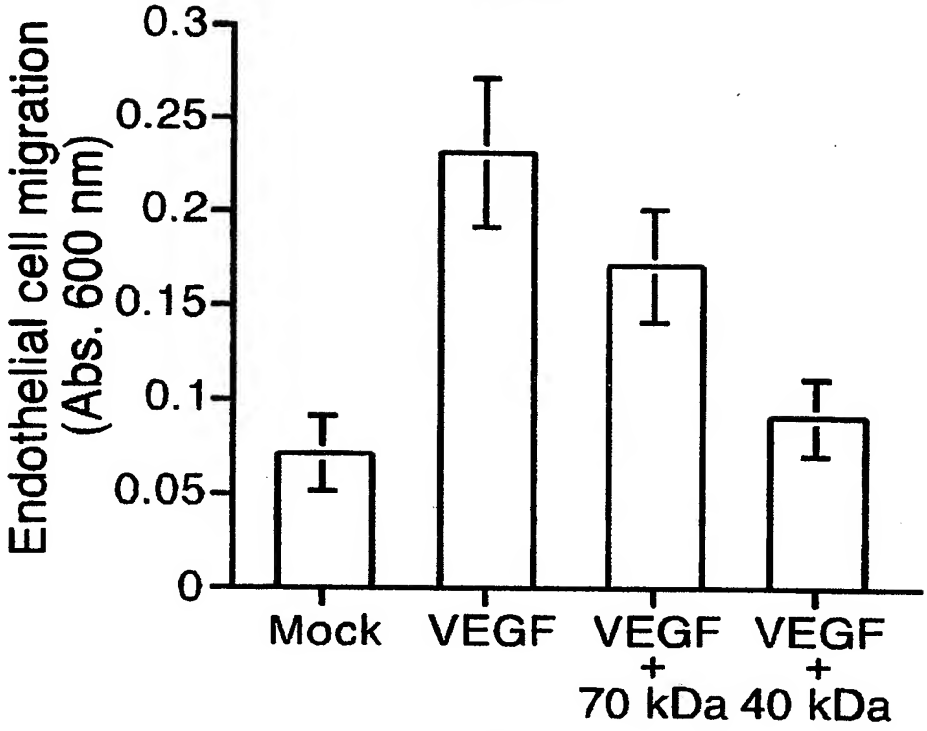

Fig. 8A

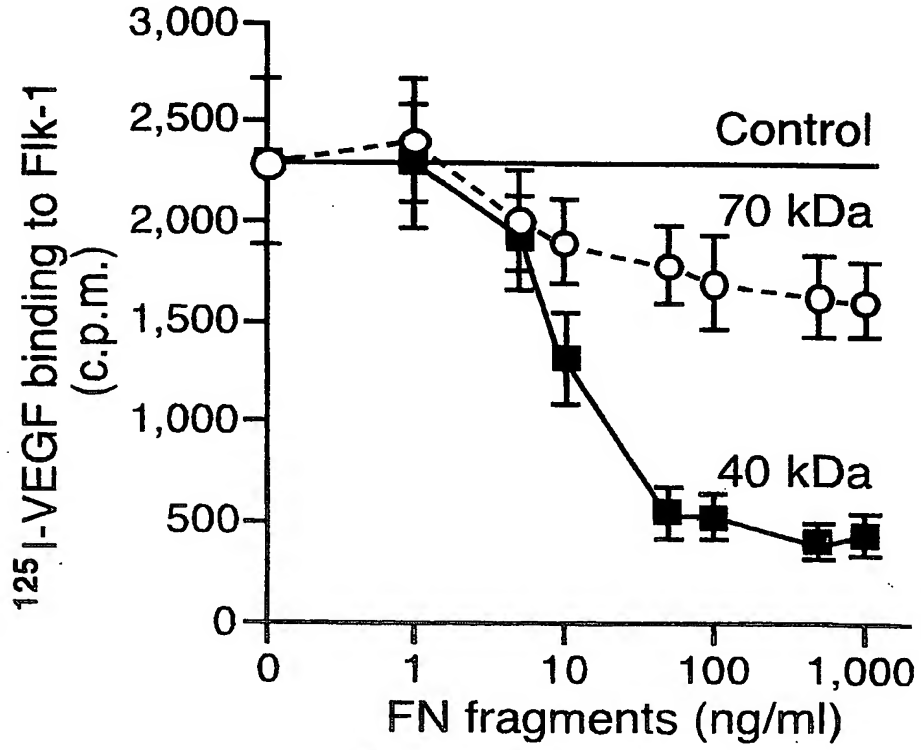

Fig. 8B

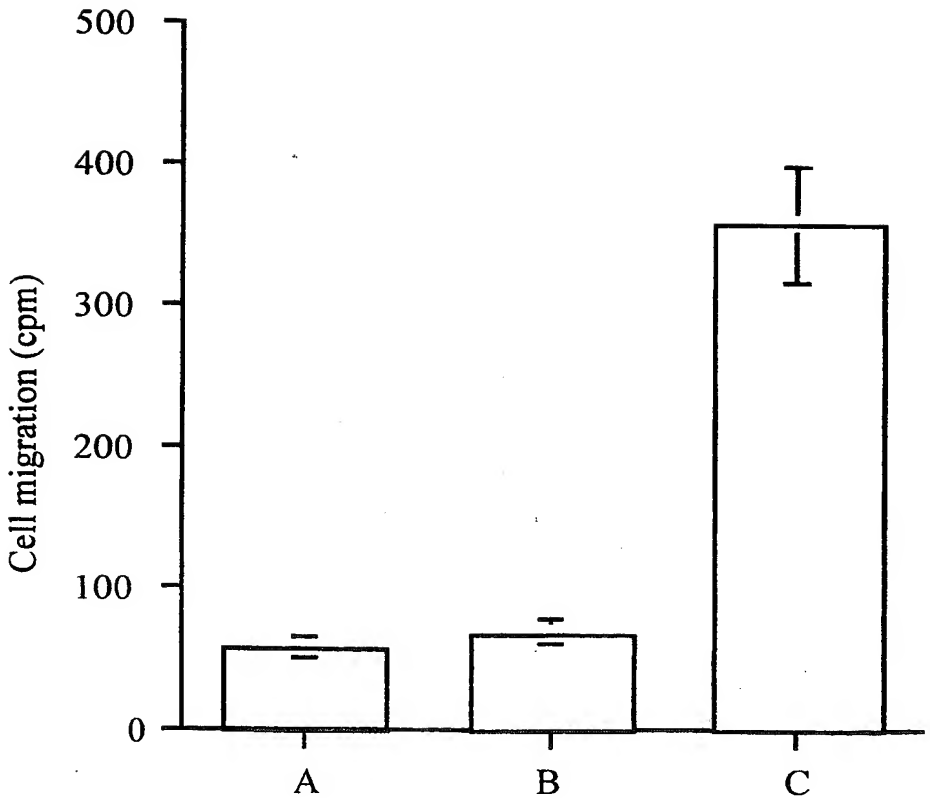

Fig. 9

SEQUENCE LISTING

<110> Wijelath, Errol S.
Murray-Wijelath, Jacqueline
Hammond, William P.
The Hope Heart Institute

<120> ENDOTHELIAL CELL STIMULATION BY A COMPLEX OF FIBRONECTIN AND VASCULAR ENDOTHELIAL GROWTH FACTOR

<130> HOPE-1-15239

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07183

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 14/78, 14/475; A61K 38/18, 38/39
US CL : 530/350, 356, 382; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 356, 382; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	JP 10-137334 A (TOA GOSEI CHEM IND LTD) 26 May 1998 (26.05.98), see entire document, especially figures and English translation of abstract.	1, 2, 19, 20, 39 and 40 ----- 3, 4, 21 and 41
Y	US 5,192,743 A (HSU et al) 09 March 1993 (09.03.93), column 2, lines 44-46 and column 9, lines 43-54.	1-4, 19-21 and 39-41
Y	US 5,652,225 A (ISNER) 29 July 1997 (29.07.97), column 7, lines 1-10.	1-4, 19-21 and 39-41

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 JULY 2000	Date of mailing of the international search report 16 AUG 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer EILEEN B. O'HARA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)☆

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07183

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specificaliy:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 19-21 and 39-41

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07183

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

East 1.1, STN/CAS, medline, biosis, embase
search terms: inventor names, VEGF, vascular endothelial growth factor, fibronection, matrix, fibrin, hyaluronic, chondroitin, heparin, polyactate, polyglycolic, starch, collagen

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-4, 19-21 and 39-41, drawn to a composition comprising an isolated complex formed between vascular endothelial growth factor and fibronectin, a method of promoting angiogenesis and a method of stimulating the migration of vascular endothelial cells using the complex.
Group II, claim(s) 5-6, 8 and 9, drawn to chimeric polypeptide comprising a peptide fragment of fibronectin and a peptide domain comprising an RGD amino acid sequence and multimers of said chimeric polypeptide.
Group III, claim(s) 7 and 14, drawn to a chimeric polypeptide comprising a peptide fragment of fibronectin, a peptide domain comprising an RGD amino acid sequence and a peptide fragment of vitronectin, and multimers of said chimeric polypeptide.
Group IV, claims 10-13, drawn to a composition comprising a complex formed between a vascular endothelial growth factor, platelet derived growth factor-BB, and the chimeric polypeptide comprising a peptide fragment of fibronectin, a peptide domain comprising an RGD amino acid sequence and a peptide fragment of vitronectin.
Group V, claims 15-18 and 38, drawn to a chimeric polypeptide comprising a peptide fragment of fibronectin and vitronectin (and lacks an RGD peptide domain), multimers of the chimeric polypeptide and a method of inhibiting a physiological response of a cell by exposing the cell to the chimeric polypeptide.
Group VI, Claims 22-27, drawn to a method of promoting angiogenesis by exposing cells to a composition comprising a complex formed between vascular endothelial growth factor and a chimeric polypeptide comprised of a peptide fragment of fibronectin and a peptide domain comprising an RGD amino acid sequence, or multimers of said chimeric polypeptide.
Group VII, claims 28-30, drawn to a method of promoting angiogenesis by exposing cells to a composition comprising a complex formed between platelet derived growth factor and a chimeric polypeptide comprised of a peptide fragment of fibronectin, a peptide fragment of vitronectin and a peptide domain comprising an RGD amino acid sequence, or multimers of said chimeric polypeptide.
VIII, claims 31-37, drawn to a method of inhibiting a physiological response of vascular endothelial cell by exposing the cell to a protein fragment from fibronectin that does not contain an RGD peptide domain.
Group IX, claims 42-43, drawn to method for inhibiting VEGF receptor function.

The inventions listing as Groups I-IX do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical feature for the following reasons: Group I corresponds to the first invention wherein the first product is the composition comprising vascular endothelial growth factor and fibronectin, and the first methods of using are use of the composition in methods of promoting angiogenesis and stimulating migration a vascular endothelial cells. Each of groups II-IX does not share the same or corresponding special technical feature because each group is drawn to different polypeptides and compositions of polypeptides and methods. This Authority therefore considers that the several inventions do not share a special technical feature within the meaning of PCT Rule 13.2 and thus do not relate to a single general inventive concept within the meaning of PCT Rule 13.1.